PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Is Dengue Vector Control Deficient in Effectiveness or Evidence?: Systematic Review and Meta-analysis

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Abstract

Background

Although a vaccine could be available as early as 2016, vector control remains the primary approach used to prevent dengue, the most common and widespread arbovirus of humans worldwide. We reviewed the evidence for effectiveness of vector control methods in reducing its transmission.

Methodology/Principal Findings

Studies of any design published since 1980 were included if they evaluated method(s) targeting *Aedes aegypti* or *Ae. albopictus* for at least 3 months. Primary outcome was dengue incidence. Following Cochrane and PRISMA Group guidelines, database searches yielded 960 reports, and 41 were eligible for inclusion, with 19 providing data for meta-analysis. Study duration ranged from 5 months to 10 years. Studies evaluating multiple tools/approaches (23 records) were more common than single methods, while environmental management was the most common method (19 studies). Only 9/41 reports were randomized controlled trials (RCTs). Two out of 19 studies evaluating dengue incidence were RCTs, and neither reported any statistically significant impact. No RCTs evaluated effectiveness of insecticide space-spraying (fogging) against dengue. Based on meta-analyses, house screening significantly reduced dengue risk, OR 0.22 (95% CI 0.05–0.93, $p = 0.04$), as did combining community-based environmental management and water container covers, OR 0.22 (95% CI 0.15–0.32, $p<0.0001$). Indoor residual spraying (IRS) did not impact significantly on infection risk (OR 0.67; 95% CI 0.22–2.11; $p = 0.50$). Skin repellents, insecticide-treated bed nets or traps had no effect (p>0.5), but insecticide aerosols (OR 2.03; 95% CI 1.44–2.86) and mosquito coils (OR 1.44; 95% CI 1.09–1.91) were associated with higher dengue risk ($p = 0.01$). Although 23/41 studies examined the impact of insecticide-based tools, only 9 evaluated the insecticide susceptibility status of the target vector population during the study.

Conclusions/Significance

This review and meta-analysis demonstrate the remarkable paucity of reliable evidence for the effectiveness of any dengue vector control method. Standardised studies of higher quality to evaluate and compare methods must be prioritised to optimise cost-effective dengue prevention.

Author Summary

Dengue fever has increased dramatically over the past 50 years and today is the most widespread mosquito-borne arboviral disease, affecting nearly half the world's population in 128 countries. Until the arrival of a vaccine, control of its *Aedes* vectors has been the only method to prevent dengue infection. With dengue outbreaks occurring at increasing frequency and intensity, we undertook a systematic review and meta-analysis of the literature, to evaluate the evidence for effectiveness of vector control strategies currently available. Forty-one studies (from 5 months to 10 years duration) were included in the review. Most studies investigated combinations of approaches but only 9 studies were randomized controlled trials (RCTs). Remarkably, no RCTs evaluated effectiveness against dengue of insecticide space-spraying (outdoor fogging), the main response to dengue outbreaks used worldwide. Nevertheless, there was limited evidence indicating that house screening and to a lesser extent, community-based environmental management with water container covers could reduce risk of dengue infection. However, skin repellents, bed nets and mosquito traps had no effect while insecticide aerosols and mosquito coils were associated with higher dengue risk. However, the quality of the few studies eligible for inclusion was poor overall, and the evidence base is very weak, compromising the knowledge base for making recommendations on delivery of appropriate and effective control. Given this paucity of reliable evidence, standardised studies of higher quality must now be a priority.

Introduction

Dengue is a viral infection transmitted between humans by *Aedes* mosquitoes. With an estimated 390 million dengue infections occurring every year, and almost half the world's population exposed to infection with dengue viruses, it is the most widespread mosquito-borne arboviral disease today, affecting 128 countries worldwide [1–3]. The dramatic increase in dengue over the past 50 years can be attributed to a number of factors, ranging from increased urbanization, in-country and international population movement, erratic water supplies and ineffective or unsustainable vector control [4, 5]. The human and economic cost of frequent dengue outbreaks is high, though current Figs are almost certainly underestimates [6–9]. Dengue is showing signs of emergence in more temperate latitudes [10–13] and is a potential threat to many of the international mass-gatherings that are a feature the modern era, such as the FIFA World Cup and the Olympics, or religious gatherings like the Hajj, although their contribution to global spread has never been proven [14, 15].

Until recent advances in vaccine development [16–17], and the approval and potential availability of the first product in 2016 [18], dengue has been unique among the major vector-borne diseases, in that prevention from infection could only be attempted by reducing or eliminating bites by infected vector mosquitoes [19, 20].

Dengue viruses are transmitted primarily by *Aedes aegypti*, a cosmotropical mosquito that thrives in urban environments. It is highly anthropophilic and breeds in small bodies of fresh water, most commonly in the numerous containers found around the home, ranging from water storage drums and overhead tanks to bottles, buckets and discarded waste items [4]. Between blood feeding and oviposition, adult female mosquitoes rest within or close to human dwellings [19]. A second vector, *Aedes albopictus*, was originally confined to Asia, but in recent decades has expanded its global range and contributed to the spread of the chikungunya virus, as well as dengue [21–24].

Control of dengue vectors can be directed against the immature aquatic stages (larvae and pupae) or the adult mosquitoes, with a number of methods available for each approach. Described in detail elsewhere [19, 25], they can be grouped according to whether they target the vector directly (*i.e.* aim to kill mosquitoes using insecticides or natural enemies or prevent them from biting using repellents) or indirectly (*e.g.* environmental modification or sanitation improvements that reduce potential larval development sites, or house improvements that prevent mosquito entry). Some approaches require skilled staff and/or dedicated resources (*e.g.* specialised spraying equipment, insecticides, transport) in order to be delivered effectively in a vertical approach. For others, affected communities, empowered through education and advocacy, can mobilize and mount effective control operations relatively independently via horizontal or community-based efforts. Hence, space-spraying and larviciding require trained personnel to deliver potentially toxic insecticides using specialized equipment and are dependent on vertical municipality-driven programs. In contrast, reductions in potential larval development sites can be achieved with householders and communities taking responsibility, supported by education and social mobilization [19].

In dengue-affected communities worldwide, immature vector populations are targeted through the reduction or elimination of potential larval development sites, typically by collection of purposeless or discarded containers in 'clean-up' or environmental management campaigns; functional or useful sites are either covered (water storage containers), drained (gutters or channels) or treated with an appropriate insecticide (usually referred to as 'larviciding') or biological control agent (predatory copepods or fish). Identification of, and targeted action towards, 'productive' container types (*i.e.* those that are assessed as contributing the greatest burden of pupae, relative to other containers in the area) can potentially enable more cost-effective larval control [26,27].

The typical response to dengue outbreaks is to target the adult mosquito population by space-spraying or fogging with insecticide, delivered outside or inside the home, with the aim of severely reducing the vector population at the time of delivery. This method is not designed to deliver persistent insecticide residues on treated surfaces and if the outbreak continues, it must be repeated at intervals that coincide with the vector life cycle [19].

Previously, Erlanger *et al.* (2008) [28] reviewed data on the effectiveness on vector indices of all vector control methods and concluded that integrated vector control was the most effective, while environmental management had minimal impact. Notably, the evidence for impact of outdoor space spraying was limited, though only 1 of the studies included was less than 30 years old (dated from 2015). Two subsequent reviews [29, 30] focused on peri-domestic space spraying and concluded that there was no evidence to support its use in dengue outbreak control, either as a standalone intervention or in combination with other interventions. Horstick *et al.* (2010) [31] also found no evidence for a demonstrable effect of vector control on entomological indices and identified specific weaknesses in funding, management, staffing and community engagement, all of which conspired to lower operational standards and ultimately restrict any likelihood of success. Recent reviews have examined the evidence for the effectiveness of individual methods, including copepods, fish and temephos [32–34].

Today, dengue outbreaks occur at an increasing frequency and intensity in affected communities worldwide and the need for evidence-based selection of the most appropriate interventions has never been greater. What are the best currently available dengue vector control tools, as measured by their impact on dengue infections, and not simply on vector populations? Are previous dengue control failures the result of low operational and management strategies, or are the available tools simply not effective? What evidence exists to provide a basis for evaluating dengue vector control today? To answer these questions and to provide guidance on the most effective strategies currently available to combat dengue, we report here on a systematic review and meta-analysis of the evidence.

Methods

Objectives

To systematically review randomized and non-randomized studies to evaluate the evidence of the effectiveness of vector control interventions in reducing a) *Aedes sp.* vector indices and b) human DENV infection and/or disease. The original search was conducted in April 2012 and updated in December 2013 and on 10th January 2015.

Eligibility criteria

Table 1 displays the eligibility criteria. Only studies that presented data for a minimum duration of 3 months were included (regardless of the frequency of treatments undertaken within that period), as this was considered the minimum period required to demonstrate a sustained impact on the vector population and/or impact on dengue transmission. In addition, only studies published since 1980 were considered eligible for inclusion, for a number of reasons. The period after 1980 saw the expansion in urban populations worldwide, notably in the less developed countries where the ratio of populations in urban and rural regions began to change dramatically [35,36]. This also was the beginning of the 'globalization' era, as characterized by steep increases in trans-national and international movement of humans and merchandise, and the time when all four dengue serotypes were reported in every continent, leading to an increase in the frequency and magnitude of dengue outbreaks [5,37,38]. We are familiar with the achievements prior to 1970, such as the ambitious yellow fever programs when *Aedes aegypti* populations were significantly diminished, and indeed eliminated from many cities and large geographic areas throughout Latin America [1,4,5,39]. On balance, it was concluded that the control tools available before the 1980s (*e.g.* the highly persistent insecticide DDT) and the settings in which they were carried out, were not pertinent to the challenge of dengue control in urban environments of the 21st century, based on the significant logistical, sociological and

Table 1. Criteria for inclusion or exclusion of studies.

	Inclusion Criteria	Exclusion Criteria
Study design	Any randomised or non-randomised study design.	Review articles or opinion papers
	Primary research and models using empirical data.	Non-empirical research/ modelled data
Mosquitoes	*Aedes aegypti/ albopictus*	All other mosquito spp.
Interventions	Any study where vector control tools (singly or combined) were used for >3 months	
Outcomes	Any study with empirical data reporting dengue incident data and/or entomological indices monitored longitudinally for the duration of the intervention	Entomological data without longitudinal (interval) data capture
	Dengue cases reported either by the study or obtained from external institutions (e.g. hospital records)	Qualitative dengue reports
Other	Papers published from 1980 onwards	Papers published pre-1980

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epidemiological changes, and the rise in insecticide resistance in vector populations [40,41] that have occurred in many of those countries during the past 35 years.

Outcomes

The primary outcome was dengue incidence (any reported case data; clinical or lab-confirmed/serologically positive cases); secondary outcomes were a range of vector indices: Breteau Index (BI), House Index (HI), Container Index (CI), tank positivity, number of mosquito adults, pupae per person index (PPPI), presence of *Aedes* immatures and ovitrap positivity rates.

All methods were pre-specified in the review protocol. PRISMA Group guidelines were followed as standard methodologies [42,43].

Search strategy

The databases WHOLIS, MEDLINE, EMBASE, LILACS and Science Citation Index were searched using the Medical Subject Heading (MeSH) "dengue" followed by the Boolean operator "and" combined with the following 'free text' terms "epidemic" and further combined in succession with: 'threshold' 'sentinel' 'early warning' 'case management' 'vector control' 'DDSS' 'space spraying' 'indoor residual spraying' 'fogging' 'integrated vector management' 'IVM' 'source reduction' 'container' 'larvicide' 'repellent' 'insecticide' 'adulticide' 'fumigant' 'aerial spraying' 'dengue decision support system'. The reference list of each of the included studies was also searched, and "grey literature" (cited unpublished documents) were sought by communication with authors. No limits were placed on year of publication status or language.

Study selection

Search results were imported into EndNote (EndNote X5, Build 7473). LRB and PJM independently assessed the title and abstract of each record (or the corresponding full article) retrieved by the search for eligibility; any discrepancies were discussed. The full article was retrieved for each eligible study. The study's investigators were contacted if eligibility was unclear, additional data were unpublished or the article was inaccessible. Each article was scrutinized to detect multiple publications from the same trial; such publications were included as a single study.

Data extraction

LRB and PJM independently extracted data according to an agreed checklist and differences were discussed. Trial characteristics and risk of bias information were extracted along with outcome data (S1 Table). For each randomized controlled trial, we extracted the number of individuals randomized and the number of individuals analysed for each treatment group. For dichotomous outcomes, we extracted the number of individuals experiencing the event in each treatment group for each study. For continuous outcomes we extracted means and standard deviations (where presented) or medians, interquartile ranges, and ranges. When such data were not reported, we extracted narrative information and tabulated results. For non-randomized studies, we extracted measures of effect, as well as treatment group data.

Risk of bias assessment

Using a pre-piloted form, LRB and PJM independently assessed risk of bias and discussed any differences (S2 and S3 Tables). For randomized controlled trials we used the Cochrane risk of bias tool and addressed: random sequence generation; allocation concealment; blinding; incomplete outcome data, selective outcome reporting, and other biases [43]. For each component of each trial, a judgment of high, low, or unclear risk of bias was made and the rationale

for the judgment was given (S2 Table and S1 Fig). For non-randomized studies, LRB and PJM used the Quality Assessment Tool for Quantitative Studies [44] (S3 Table). This ensured that each study could be ranked according to inherent study design limitations, which included but were not limited to, bias, confounding and blinding.

Data analyses

Analyses were performed in Review Manager (RevMan Version 5.2. Copenhagen: The Nordic Cochrane Centre, 2012). We extracted the measure of effect and CI from the study reports. Where possible, we stratified analyses by intervention, outcome, measures of effect and study design. For multi-arm trials, data from numerous intervention groups were pooled. We calculated trial-level results (*i.e.* MD, RR or OR and standard error [SE]) and pooled them using random-effects inverse-variance meta-analysis to account for large variability present between studies. Results were visualised in forest plots. Sub-group analyses were used to stratify studies that used different and/ or combination interventions.

Heterogeneity was assessed using the I^2 test statistic, the chi-squared test ($P<0.01$ indicated possible significance) and by visual inspection of the forest plots to identify overlapping confidence intervals. Studies that could not be visualised in forest plots were presented in tables.

When heterogeneity was detected, possible causes were explored using subgroup analyses and predefined covariates.

Subgroup analyses were planned to explore potential sources of heterogeneity (*i.e.* effects of seasonality, mosquito species, duration of intervention, coverage), but analyses were not carried out because of the low number of studies available for analysis. For the same reason, sensitivity analyses that excluded studies with a high risk of bias were pre-planned to assess the robustness of results, but were not carried out. Hence, the planned funnel plots were not constructed to explore possible publication biases.

Results

Study eligibility results

A total of 960 potentially relevant studies were identified using systematic searches of the databases, grey literature and their cited reference lists and 19 more were identified from other sources (Fig 1). After removing duplicates, 582 citations were screened, of which 480 were excluded. The full texts of the remaining 102 records were assessed and 61 articles were excluded.

The reasons for exclusion were: incomplete outcome data (18 studies); study was a review, a non-peer reviewed report or a mathematical model (14 studies); no intervention was carried out (eight studies); undefined or inadequate dengue case definition (three studies); intervention or outbreak duration was less than 3 months (10 studies); study included only one required outcome (three studies); study preceded 1980 (three studies); time series data collection not reported (two studies).

Forty-one studies were included in the review [45–85] (S1 Table), nineteen of which reported sufficient data for inclusion in meta-analyses [46–48, 52, 54, 55, 58, 59, 66, 69, 73, 74, 76, 77, 80–83, 85].

Characteristics of included studies

The main characteristics of included studies are summarised in S4 Table. Of the 41 included studies, geographic study locations comprised: SE Asia (n = 11) or Central America (10), South

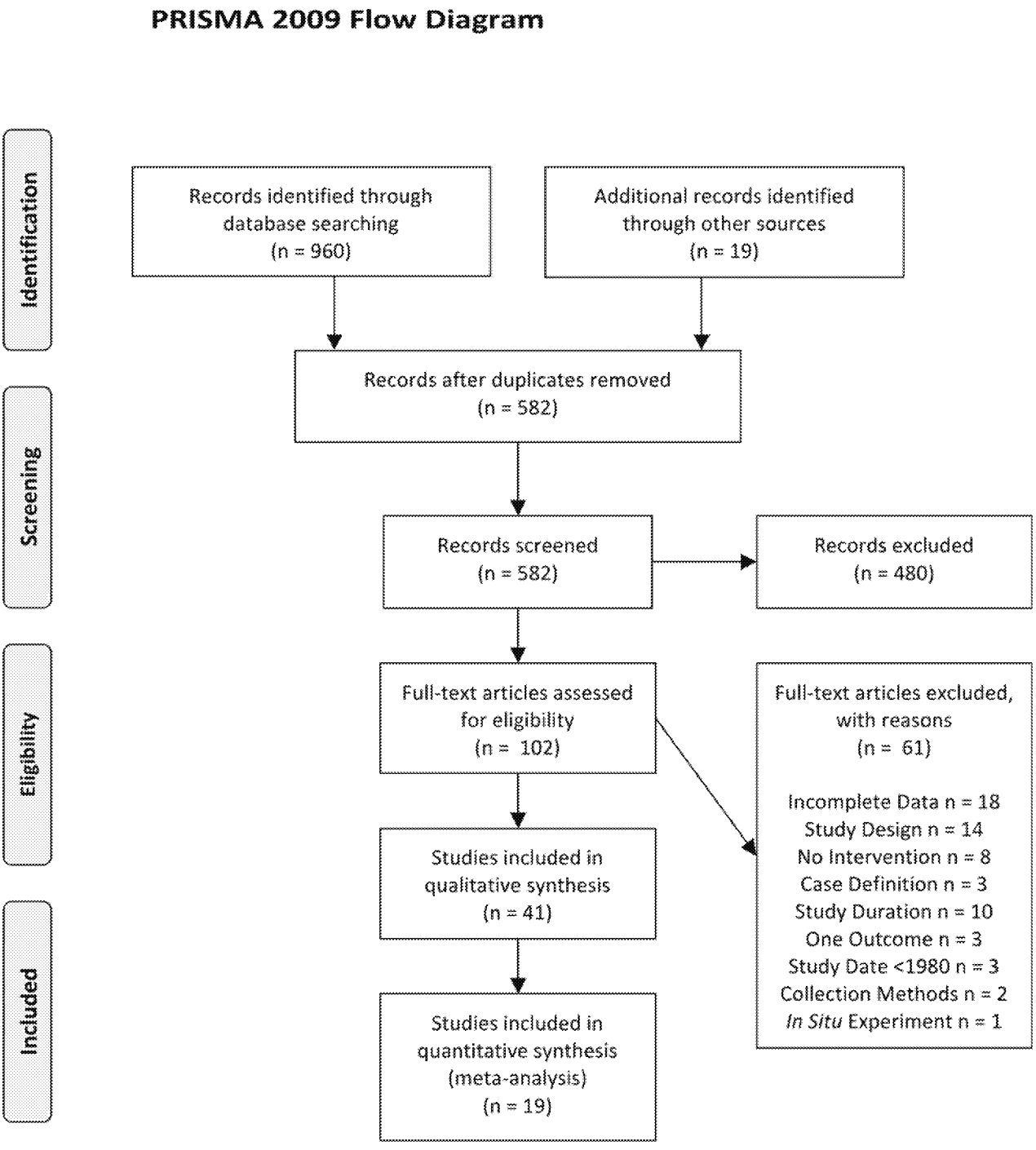

Fig 1. PRISMA 2009 flow diagram. Diagram of searches performed and the number of articles returned and examined at each stage.

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Asia (8), Australasia (4), South America (5) and North America (3). All studies were published between 1986 and 2014, and 2009 was the median year of publication.

Grouped by study design, the studies comprised: 9 randomised controlled trials (*i.e.* 7 cluster-randomized and 2 randomized controlled trials) and 32 non-randomised studies (*i.e.* 8 controlled trials, 7 longitudinal studies, 4 interrupted time series studies, 5 before and after studies,

2 observational studies, 1 case-control study, 1 cross sectional study, 1 retrospective observational study, 1 ecological study and 2 models) (S4 Table).

Vertical and community-led interventions were used exclusively in 20 and 10 studies respectively, and 11 studies used a combination of both. Combination interventions (23 studies) were more common than single interventions (18 studies). Study duration ranged from 5 months to 10 years; 16 studies were less than 1 year, 12 took place over 1–3 years and 7 studies were 8 or more years in duration.

Fig 2 (top) summarises the frequency of vector control tools by study design. The most frequently evaluated interventions were clean-up programs (n = 19), of which 4 were cluster randomised controlled trials. Outdoor fogging (9), education (11), larviciding (7) water jar covers (7) also were the subject of multiple studies.

All studies presented data on *Aedes aegypti*; four presented additional data on *Aedes albopictus* (S4 Table). Nineteen studies reported dengue incidence, 17 studies reported BI, 16 studies reported HI, 11 studies reported CI, 1 study reported tank positivity, 3 studies reported number of mosquito adults, 6 studies reported pupal indices, 3 studies reported ovitrap data.

Fig 2 (bottom) summarises the reported reduction in outcome at a statistically significant level ($p<0.05$). Of note was the observation that in studies where it was an outcome, dengue incidence was not reduced in either of 2 randomised study designs, although 8 of 14 studies with other experimental designs reported a statistically significant reduction.

Risk of bias assessment results

Randomised studies. The results of this assessment are presented in S2 Table and S1 Fig. Nine studies were at low risk of bias for selective outcome reporting. Seven studies were at low risk of bias for incomplete outcome data, while one was at medium risk and one was at a high risk of bias. There was a high risk of bias due to inadequate blinding in all studies. Risk of bias through allocation concealment was low in one study, unclear in four studies and high in four studies. Risk of bias attributed to generation of allocation sequence was low in four studies, unclear in four studies and high in one study.

Non-randomised studies. The results of this assessment for non-randomised studies are shown in S3 Table. Nineteen studies scored 3, equating a weak study, while nine studies scored 2, equal to a moderately strong study, and only two studies scored 1, equal to a strong study.

Effectiveness of interventions

Nineteen studies [46–48, 52, 54, 55, 58, 59, 66, 69, 73, 74, 76, 77, 80–83, 85] provided sufficient data to allow their inclusion in meta-analyses. The results of those analyses are presented here stratified by reported outcome, either the impact on dengue incidence or on vector indices.

Impact on dengue incidence

Impact of dengue incidence in randomised controlled trials. None of the included reports that investigated the impact of vector control on dengue incidence were randomised controlled studies.

Impact on dengue incidence in non-randomised controlled trials. Five studies measuring the impact of any intervention[s] on dengue incidence using odds ratios were included in one meta-analysis (Fig 3). These included a number of study designs (cross sectional, observational [x2], retrospective observational, case-control) and interventions (knockdown sprays or insecticidal aerosols, house screening, indoor residual spraying, community-based environmental management, insect repellents, bed nets, mosquito coils and mosquito traps).

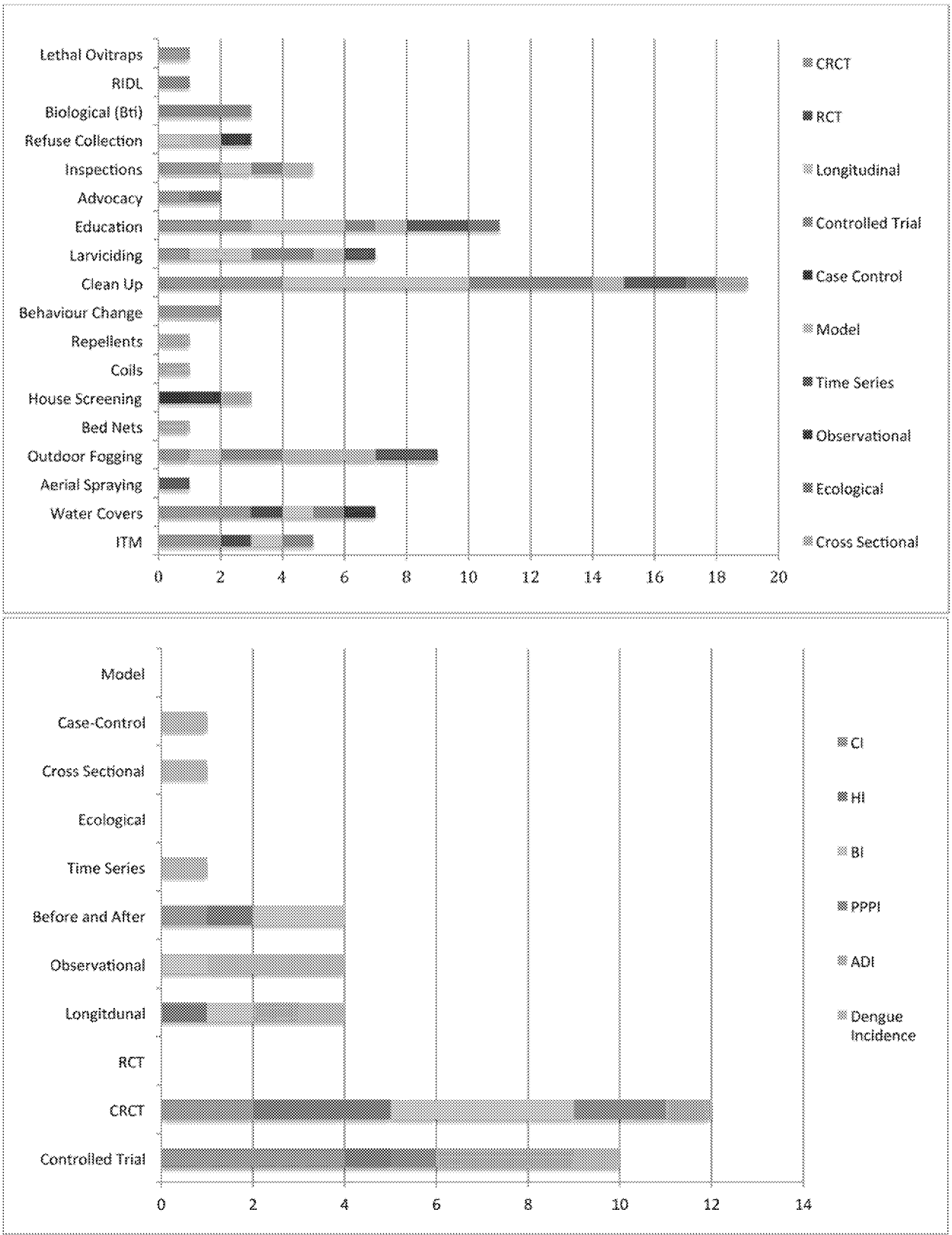

Fig 2. Summary of vector control tools and approaches. Top: Histogram of frequency of interventions reported by the 41 studies, stratified by study design (note that a study design may have evaluated more than 1 intervention). Bottom: Histogram of frequency of reported reductions at $p<0.05$ stratified by study design (ADI = adult (mosquito) density index; CRCT = cluster randomised controlled trial).

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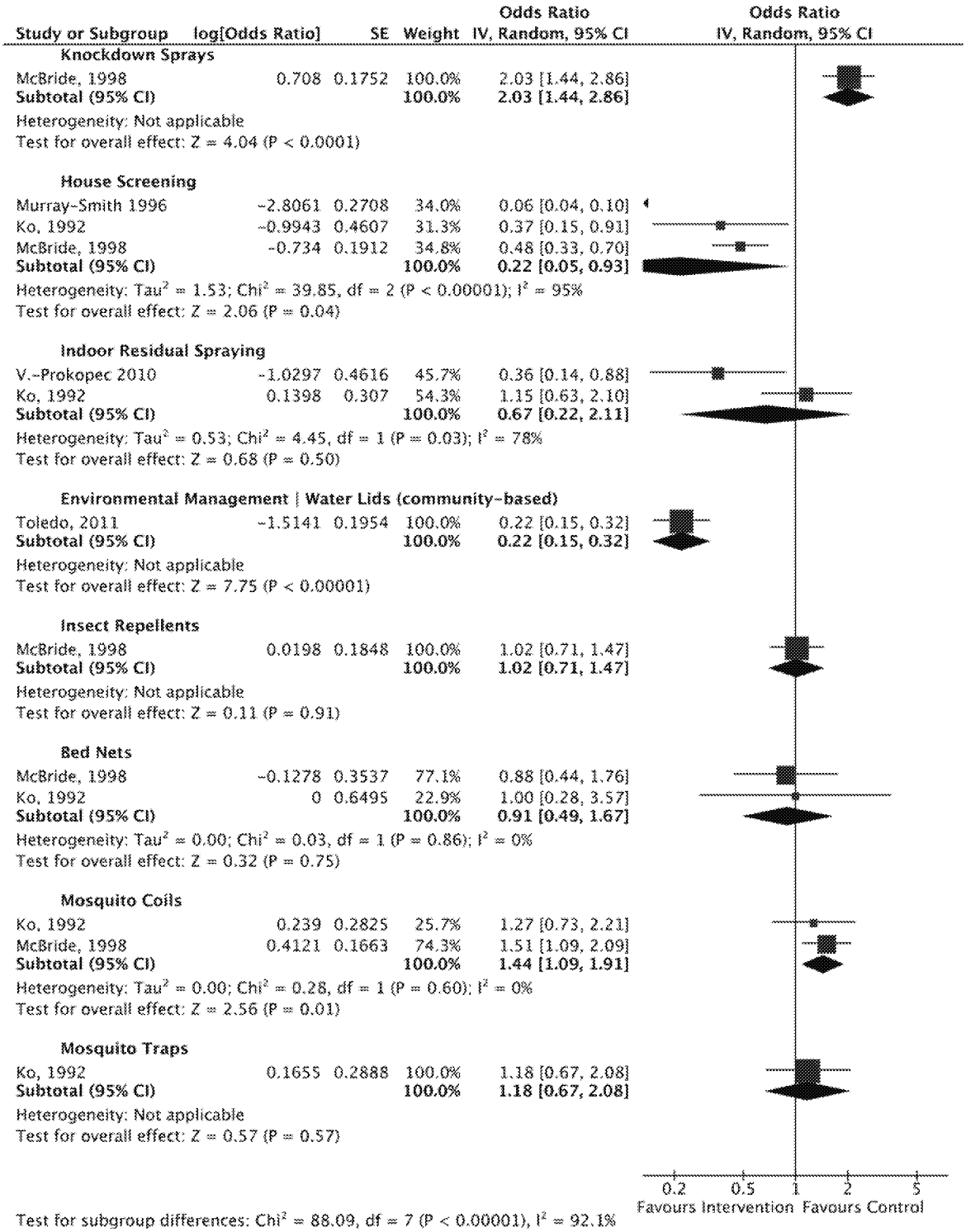

Fig 3. Forest Plot of comparison: Non-randomised controlled trials sub-group analysis stratified by intervention vs. control, for the outcome dengue incidence. *NOTES*: Toledo (2011)[58], original risk ratio was assumed to be similar to the odds ratio, which may bias in favor of the intervention; McBride (1998)[52] cross-sectional study design with no control group); insect repellents upper confidence limit was corrected from 1.44 to 1.47 by RevMan; Ko (1992)[69]: mosquito traps, upper confidence limit was altered by Revman from 2.05 to 2.08; mosquito coils, upper confidence limit altered by RevMan from 2.22 to 2.21; house screens, confidence limit altered by RevMan from 0.89 to 0.91. Vasquez-Prokopec *et al*. (2010)[66], IRS odds ratios relate to secondary dengue infections only.

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Heterogeneity across the studies was high, most likely due to the varying study designs, number of studies per subgroup and intervention type (I^2 = 92.1%).

The presence of house screening in homes (three studies: 52,59,69) significantly reduced the odds of dengue incidence compared to homes without screens (0.22: 95% confidence interval (CI) 0.05, 0.93; p = 0.04). Combined community-based environmental management together with the use of water container covers [58] also reduced the odds of dengue incidence to 0.22 (95% CI 0.15, 0.32; p<0.0001).

Indoor residual spraying reduced the odds of infection to 0.67 (95% CI 0.22, 2.11), but the result was not significant (p = 0.50) [66,69]. There was no evidence that the use of mosquito repellents [52], bed nets [52,69] or mosquito traps [69] significantly increased or reduced the odds of dengue infection, with odds ratios of 1.02 (95% CI 0.71, 1.47; p = 0.91), 0.91 (95% CI 0.49, 1.67; p = 0.75) and 1.18 (95% CI 0.67, 2.08; p = 0.57) respectively.

Conversely, the use of knockdown sprays [52] (OR 2.03; 95% CI 1.44, 2.86) or mosquito coils [52,69] (OR 1.44; 95% CI 1.09, 1.91; p = 0.01) was significantly associated with an increased odds of dengue incidence.

Impact on vector indices

Impact on mosquito indices evaluated in cluster-randomized controlled trials (CRCTs). Cluster-randomized controlled trials with data suitable for inclusion in these -analyses investigated: insecticide-treated curtains (ITCs) [76, 77]; community-based combination interventions such as waste disposal, clean up campaigns, formation of community working groups, mobilization and education [73]; source reduction, larviciding, entomological surveillance, communication, education and punitive fines [47]. Forest plots of analyses measuring impacts on the BI, HI, CI and pupal indices are shown in Figs 4 and 5.

As shown in Fig 4, ITCs [76, 77] did not significantly reduce the pooled mean difference for either BI, (-25.16; 95% CI -76.02, -25.70; p = 0.33), HI (-10.58; 95% CI -32.22, -11.05; p = 0.34),

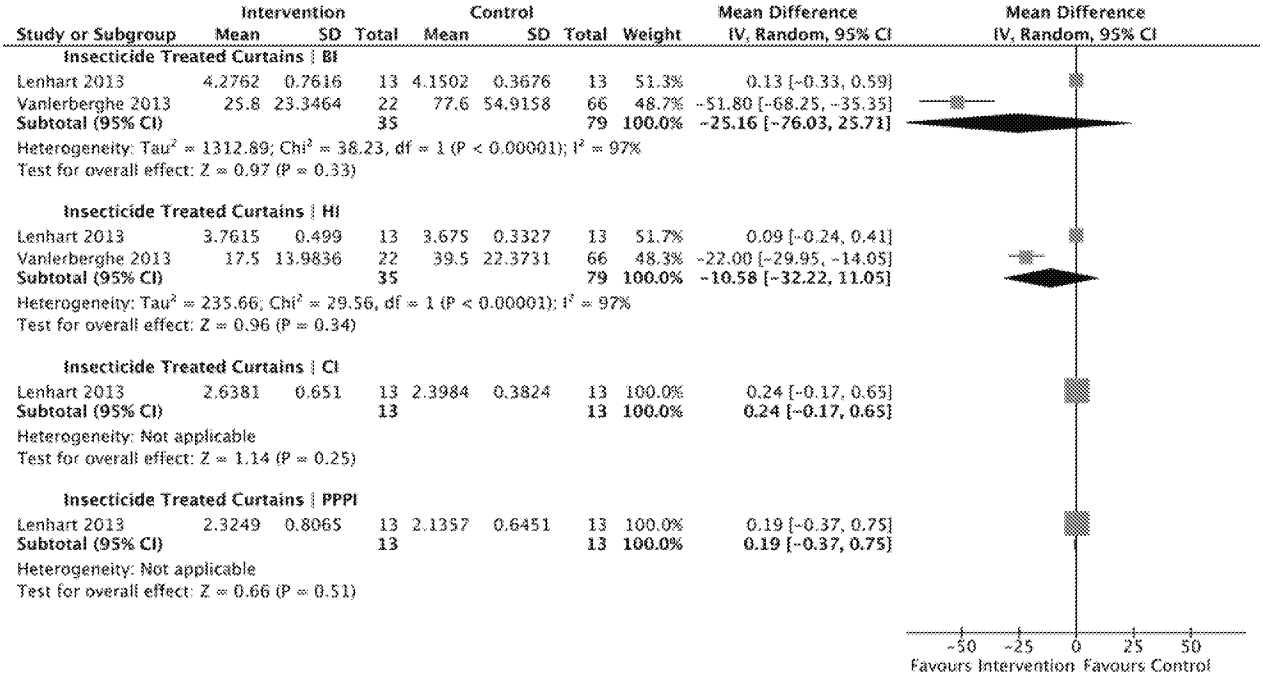

Fig 4. Forest plot of comparison: Cluster randomised controlled trials sub-group analysis for insecticide-treated curtains intervention vs. control for the outcomes Breteau Index, House Index, Container Index and Pupae Per Person Index.

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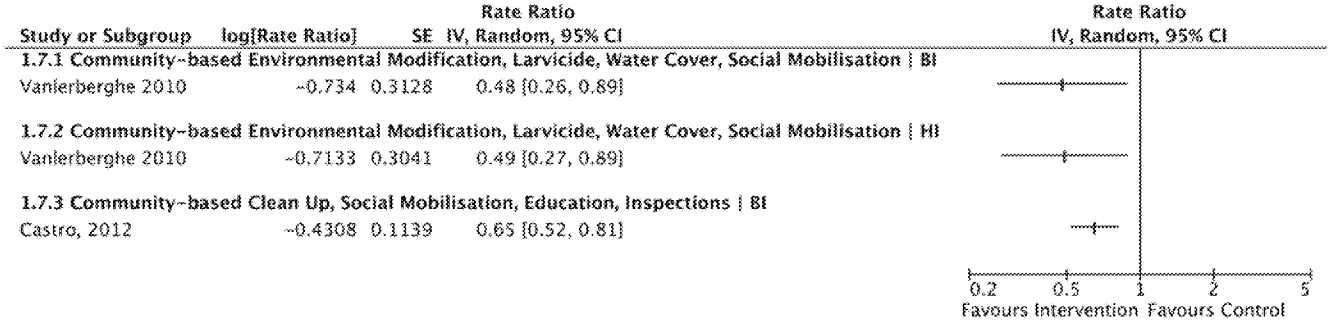
CI (-0.24; 95% CI -0.16, 0.25) or Pupal indices (-0.19; 95% CI -0.37, 0.75). Heterogeneity between the studies was high, with I_2 = 97% ($p<0.0001$) for outcomes BI and HI.

In Cuba, community-based combination interventions significantly impacted BI and HI (Fig 5), with rate ratios of 0.48 (95% CI 0.26, 0.89) and 0.49 (95% CI 0.27, 0.89) in one study [47], while another [83] found that routine interventions led by the community were significantly more effective than routine interventions alone (RR 0.65; 95% CI 0.52, 0.81). Similarly, in an 'eco-health' study in India [73], the mean difference was significantly reduced for all metrics: BI -4.66 (-5.89, -3.43), HI -17.10 (-22.16, -12.04) and CI -12.30 (-15.31, -9.29).

Impact on mosquito indices evaluated in randomized controlled trials (RCTs). One study investigated the impact of covering productive larval development container types (S2 Fig). Water tank covers significantly reduced the number of tanks positive for immature stage *Ae. aegypti* MD = -4.00 (95% CI -4.96, -3.04) [54], but an impact on dengue incidence was not evaluated.

In quasi-experimental design, larviciding using the insect growth regulator Pyriproxyfen delivered as part of a community-based strategy, was reported to have significantly reduced the rate of dengue incidence in the intervention group: RR 0.19 (95% CI 0.12, 0.30) (S3 Fig) [80].

A.



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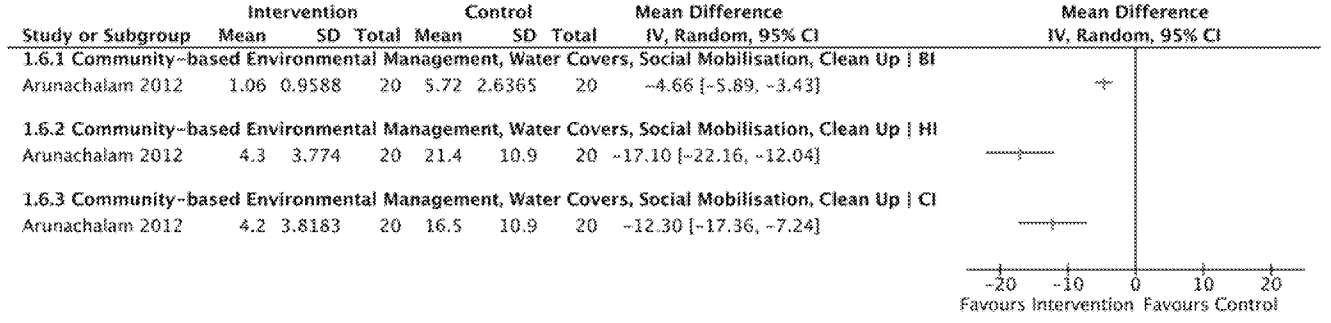

Fig 5. A. Forest plot of comparison: Cluster randomised controlled trials analysis of community-based environmental management intervention vs. control for the outcomes Breteau Index, House Index. Cluster Randomised Controlled Trials of community empowerment with routine control vs. control (routine control alone), for the outcome Breteau Index. B. Forest Plot of Comparison: Cluster Randomised Controlled Trials community-based analysis of environmental management intervention vs. control for the outcomes Breteau Index, House Index and Container Index.

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Impact on mosquito indices evaluated in non-RCTs. Although numerous studies evaluated the impact of combinations of interventions on vector populations, it was not possible to combine these into one forest plot, because of the wide range of study designs, outcomes or outcome measures applied. Variously, the studies investigated: clean-up campaigns in conjunction with IRS and larviciding [46]; community-based environmental management (including community-based environmental management, source reduction, larviciding, education, promote formation of CWGs, water covers) [48]; source reduction, larviciding and fogging [55]; nocturnal outdoor fogging [74]; the larval growth inhibitor pyriproxyfen, used alone [80] or in combination with insecticide-treated water covers [82]; lethal ovitraps [81]; release of genetically modified mosquitoes (Release of Insects with Dominant Lethality, RIDL) [85].

Community-based environmental management significantly reduced the House Index: MD = -2.14 (95% CI -3.72, -0.56) [48] (S4 Fig) and combination interventions (clean-up campaigns in conjunction with IRS and larviciding) reduced ovitrap positivity MD = -10.30 (95% CI -12.80, -7.80) [46] (S4 Fig). The use of fogging, source reduction and larviciding resulted in lower odds of detecting increased larval densities (S5 Fig): Breteau Index OR = 0.15 (95% CI 0.10, 0.24) and House Index OR = 0.13 (95% CI 0.08, 0.22) when compared to baseline [55], while the odds of the presence of immature stage *Aedes* were reduced in the intervention group, through the combined use of Olyset net covers for waters jars and pyriproxyfen for a period of 5 months (S2 Fig) [82]. Biogents Sentinel lethal ovitraps demonstrated potential in reducing the number of circulating adult mosquitoes, although this result was modest and not significant: MD 0.30 (95% CI -0.74, 0.13) (S6 Fig) [81].

Outdoor nocturnal ultra-low volume fogging significantly reduced numbers of adult *Ae. albopictus* in the intervention group by -13.90 (95% CI -21.86, -5.94) (S4 Fig) but did not measure effects on immature stages [74]. Sampling was conducted using BioGents Sentinel Trap and fogging was conducted between 3–5 times per annum; 43–90% mosquito control was achieved.

In a seminal field study with genetically-modified mosquitoes in the Cayman islands, scheduled releases of sterile male mosquitoes reduced the odds of ovitrap positivity in intervention clusters compared with control clusters: OR 0.11 (95% CI 0.07, 0.18) by [85] (S7 Fig).

Discussion

The dramatic growth in dengue over the past 35 years has been a remarkable epidemiological event and, as evidenced by its continued global spread, a challenge for which the public health community was not prepared. It is not surprising that 24 of the 41 studies included in this review were published in the past 7 years, reflecting the increase in attention and resources devoted to devising effective control strategies as recognition of the dengue pandemic grew. However, the fact that the global increase in focus on dengue control generated so few studies performed at a standard required for inclusion in this review, indicates that the magnitude of the response to the dengue pandemic has not been sufficient. Moreover, most of these studies investigated the impact of interventions on dengue vector indices alone, rather than dengue incidence. This also is discouraging, as the limitations of the Stegomyia larval indices, primarily their poor correlation with dengue transmission, are well known [86]. Finally, the inadequacy of the response to global dengue threat is demonstrated by the identification of thirteen studies that measured the impact of vector control on dengue incidence in the past 35 years, and that only six of these were suitable for inclusion in a meta-analysis. Simply stated, we do not have a clear understanding of which of the currently available interventions actually work, where or when they succeed or might work best, and the reasons why they succeed or fail.

Nowhere is the inadequacy more apparent than in the absence of appropriately designed trials to evaluate insecticide fogging or space-spraying for the prevention of dengue transmission. Although space spraying is the standard public health response to a dengue outbreak worldwide, and is recommended by WHO for this purpose [19], our study revealed the scant evidence available from studies to evaluate this method sufficiently. Earlier reviews also noted this serious omission from the literature published before 1980 [29,31]. Remarkably, no randomised controlled trials have been undertaken to evaluate the effectiveness of space-spraying or fogging to reduce dengue transmission or dengue incidence, anywhere in the past 35 years. We identified only one study [74] suitable for inclusion in a meta-analysis that demonstrated a significant impact of outdoor fogging on dengue vector populations.

Without adequate evidence, it is impossible to determine how effective space-spraying programs, whether indoor or outdoor, have been. It may be the case that outdoor fogging has the potential to impact on dengue vector populations sufficiently to impact transmission, but the minimum treatment frequency and geographic area requiring treatment remain unknown. The most encouraging report comes from a recent longitudinal study analysing twelve years of data from the city of Iquitos in Peru [84], which concluded that dengue cases could be reduced if intensive city-wide space-spraying (outdoor fogging) was conducted early in the transmission season. Given the cost implications of delivering a similar scale treatment in an even larger city, possibly with the need to do so in advance of an outbreak crisis, further studies to demonstrate the potential benefits are essential.

Of those that could be assessed adequately, the method with the most evidence supporting effectiveness in preventing dengue transmission was house screening. Data from cross-sectional [52] and case-control studies [59] in Australia, and a case-control study in Taiwan (69) were included in a meta-analysis that indicated a significant protective effect of window and door screens on dengue transmission as detected by serology (ELISA or HIA (haemagglutination inhibition assay)) (Fig 3). Although the weaker study designs limited the power of this result, the results are encouraging. *Aedes aegypti* exhibit predominantly indoor resting and blood feeding behaviour (termed endophagic and endophilic behaviour, respectively)[87], and barriers to access would be expected to impact on this species. Malaria vector mosquitoes and other arthropods of medical importance are also active indoors and can be targeted in the same way, increasing the likelihood of perception of benefit and adoption by householders. "Mosquito-proofing" houses was first considered over a century ago, and its potential as a sustainable and effective tool for malaria control has been evaluated in randomized controlled trials in recent years [88–90]. New investigations of screening for dengue prevention are also underway. Recent studies in a high-risk dengue setting in Mexico reported that window and door screens were a popular and widely-adopted intervention that significantly reduced domestic infestations of *Aedes aegypti* [91, 92]. House screening is not included in the current WHO dengue guidelines, but given its potential and wide ranging benefits, it is a strong candidate for randomised controlled trials to evaluate its effectiveness in preventing dengue.

Two observational studies reported on the impact of indoor residual spraying IRS, with contradictory results and while one of these reported a positive significant reduction in the odds of (secondary) incidence [66], the second study reported an insignificant increase [69]. Consequently, the pooled odds ratio showed no statistically significant effect between intervention and control groups. While indoor residual spraying can target *Aedes aegypti*, such methods have rarely been used, nor are currently recommended [19, 93, 94]. Yet IRS is already used widely to control a number of other vector-borne diseases in various settings worldwide and, as it allows the delivery of a range of different insecticide classes, it can be an important tool for managing insecticide resistance [95–98]. The possibility that existing IRS programs might be expanded with minimal change to include dengue is an attractive prospect.

Probably the most widespread practices to suppress dengue vector populations are clean-up campaigns, typically community-driven and in tandem with education and health promotional campaigns as well as numerous additional approaches. Efforts promoting environmental and peri-domestic clean-up to reduce vector larval development sites have been routine practices in many dengue-endemic localities for decades and as shown in Fig 2, they were the most common intervention evaluated in the reviewed studies. However, clean-up campaigns were evaluated only as one element within multiple interventions or they continued to be promoted as a background across all the arms within a study. Thus, source reduction or clean-up campaigns were applied in some way in 20 studies but were associated with interventions ranging from fogging or water container covers targeting adult mosquitoes to larviciding and copepods for control of immatures (S3 Table). Hence it is not possible to dissect their specific contribution to reducing vector populations or their impact on dengue transmission. Of these, the strongest evidence (Fig 3) was from Cuba [58] where results indicated that community working groups (CWGs), initially set up some years earlier, in a preceding study [71] promoting environmental management, conversion of garbage zones into gardens, water pipe repairs and the use of water container covers not only reduced vector indices, but also impacted dengue transmission, significantly more than the routine *A. aegypti* control programme. Although WHO recommends community participation as an essential element of sustainable dengue prevention [99], there is little evidence that it can impact on dengue transmission [100]. A number of randomised controlled studies have demonstrated significant impacts on vector indices [47, 48, 73, 83, 101] (Fig 5) even though the methods of intervention varied considerably between the studies. Results from a cluster randomised controlled trial in Nicaragua and Mexico [102] reported reductions in dengue sero-conversion rates and self-reported dengue cases as well as vector indices, following community mobilisation to deliver pesticide-free vector control. Clearly further evidence is needed. It remains to be determined how best practice is defined in any setting (*i.e.* which tools or methods the community should employ), and what coverage is necessary in order to not simply reduce mosquito indices, but to impact on dengue virus transmission.

The use of fish and crustaceans as biological control agents that prey on or compete with the immature vector stages may have potential in certain contexts, but we identified only three studies that evaluated copepods (aquatic Crustaceans) [78, 79, 103]. In all cases, the crustaceans were used together with clean-up programs, obscuring the impact of each method, and none of the reports provided sufficient data to be included in a meta-analyses. Consistent with earlier specific reviews [32, 34], there remains little evidence to suggest that biological control has widespread potential.

A substantial number of reports demonstrated impacts on vector indices of insecticide-treated materials (ITMs), deployed as window or door curtains [54, 75, 77, 82, 104, 105], although they were effective only where houses with fewer and smaller windows and doors [75–77, 104] and where coverage of the intervention was particularly high [77]. Hence, in the meta-analyses, no significant impact on vector populations was indicated and the heterogeneity between the studies was high (Fig 4). Effects on dengue incidence of ITMs used as vertical window or door screens or as horizontal covers for water containers, need to be quantified in locations and contexts where housing conditions indicate suitability. ITMs, used as curtains hung or fixed tightly across external windows and doors, function in a similar way to mesh screens, and potentially could provide enough protection without the need for insecticide, as suggested by a study in Mexico, where ITMs reduced vector populations even though the targeted population was highly resistant to the insecticide used [90].

There was no evidence of any impact on dengue infection risk by insecticide-treated bed nets [52,69], mosquito traps [69, 81] or mosquito repellents [52]. Ongoing studies are investigating a range of novel trap designs for *Aedes spp.* surveillance and control [106–108] but to

date, evidence of traps preventing any mosquito-borne disease remains elusive. Both opinion and evidence are weighed against the use of skin repellents for prevention of vector-borne diseases [109], and attention has moved towards a new generation of spatial repellents, to be deployed within or close to houses to prevent mosquito entry, possibly in combination with attractant lethal traps in what is termed a 'push-pull' strategy [108, 110].

The significant negative associations found between the use of insecticide aerosols [52] and mosquito coils [52,69] and higher odds of dengue incidence have a number of possible explanations. These tools may have been purchased in response to an actual increase in mosquito numbers, or a dengue case in the home or a neighbour's house, during a period of dengue transmission. Alternately, householders using aerosols or coils may have relied solely on these anti-mosquito devices and not have adopted any other more effective preventative measures.

Approaches involving the use of genetically modified (GM) mosquitoes or the intracellular symbiont *Wolbachia* [111] are recent advances in insect control and only one field trial, demonstrating impact on the vector population only [85], was included in this review. An increase in the numbers of reports from ongoing new trials can be expected, although the use of GM mosquitoes for dengue control will have to confront or overcome additional regulatory or ethical challenges and requirements prior to field tests and eventual deployment [112–116].

Regarding trials of methods that require the use of insecticides, we noted that while 23/41 studies examined the impact of insecticide-based tools, only 9 of these cited recent information on insecticide resistance or referred to an evaluation of the susceptibility status of the target vector population at any stage of the study. Resistance to DDT, pyrethroids and other insecticides has been documented widely in dengue vectors, and continues to emerge, potentially impacting on intervention effectiveness [40, 117–119]. Clearly, insecticide susceptibility testing must be an integral part of any trial where insecticide-based interventions are under evaluation, as recommended by the World Health Organisation [4].

Today, there is a widespread perception that *Aedes aegypti* control 'has failed' or that existing methods will not reduce dengue transmission, and that this is why we should abandon existing approaches and invest in or pursue alternative strategies [111, 120, 121]. As we have shown in this review and meta-analysis, this is incorrect. In reality, there is very little reliable evidence from appropriately designed trials to reach a conclusion about any of the control methods available. That this also applies to insecticide space-spraying or fogging illustrates clearly the urgent need for such fundamental trials.

Care in designing studies is critical. Randomized controlled trials are the most robust design for evaluating the effectiveness of any intervention [122]. In our review, only eight of the nineteen reports included in the meta-analysis (7 CRCTs, 1 RCT) were randomised, none of which reported a significant impact on dengue incidence. In contrast, eight other studies that reported a positive reduction in dengue incidence at $p<0.05$, were not derived from randomised controlled trials, but from weaker experimental designs (see Fig 3). Weakness in the designs of trials investigating vector control tools have been recognised, and expert guidance, identification of challenges and pitfalls and clear recommendations for improvement are available [123,124].

Also apparent from this review is the large number of studies investigating impacts on the vector population alone, with no measures of the effectiveness of the intervention on dengue transmission. We recognise that detecting dengue viruses or confirming current, recent or historic dengue infections are not simple routine or inexpensive tasks, requiring skills and equipment that are not available without considerable investment. However, without this additional investment, the value of many studies that are limited to evaluating impacts on the vector alone is seriously reduced. Demonstration of impact on vector populations is achievable and often reported but is no guarantee that an intervention will translate into a reduction in dengue transmission [125, 126]. This is particularly true for dengue, where the complex relationship

between vector abundance, virus transmission and human infection rates are far from clear [86,127,128].

As well as their role in dengue transmission, *Aedes aegypti* is the main urban vector of yellow fever in Africa and South America, and this species and *Aedes albopictus* variously are vectors of the Chikungunya and Zika viruses, two emerging human pathogens that constitute a new global threat [129–132]. Despite the fears surrounding these threats, the urge to respond must be tempered by reality, and based on sound evidence. In the large urban zones where these vectors proliferate, to simply continue to use what has always been used, for that reason alone, or to pursue new approaches without sound supporting evidence would be wrong, and potentially a profligate waste of resources. Hence, there is an argument for instituting a global independent advisory body to guide decisions regarding the selection of approaches and tools for control or prevention of infections transmitted by urban *Aedes sp.* vector populations, and the design of appropriate multi-centre trials to evaluate their effectiveness. With this in mind, we hope that the findings of this review and meta-analysis will contribute to the sound evidence base on which that approach would be founded.

Supporting Information

S1 Checklist. PRISMA checklist.
(PDF)

S1 Table. Data extraction summary for reviewed studies.
(XLSX)

S2 Table. Cochrane table of bias for randomized controlled trials.
(XLSX)

S3 Table. Assessment of the validity of reviewed studies: Table of bias and QATQS (quality assessment tool for quantitative studies).
(XLSX)

S4 Table. Data extraction table for all reviewed studies.
(XLSX)

S1 Fig. 100% stacked graph of Cochrane Table of bias results.
(TIFF)

S2 Fig. Forest plot of comparison: Randomised controlled trials of net covers on water storage tanks vs. control for the outcome tank positivity.
(TIF)

S3 Fig. Forest plot of comparison: Quasi-experimental study on community participation using pyriproxyfen vs. control for the outcome dengue incidence.
(TIF)

S4 Fig. Forest plot of comparison: Non-randomised controlled trials subgroup analysis for multiple interventions vs. control for the outcomes BGS Adult Catch, Breteau Index and ovitrap positivity.
(TIF)

S5 Fig. Forest plot of comparison: Non-randomised controlled trials of multiple interventions vs. baseline for the outcomes Breteau Index, House Index. Controlled trial subgroup analysis for larvicide, ULV/ source reduction and Olyset container covers and pyriproxifen vs.

control, for the outcomes HI, BI and presence of *Aedes sp*. immatures stages.
(TIF)

S6 Fig. Forest plot of comparison: Cluster randomised controlled trials sub-group analysis for BioGents Sentinel Trap vs. control for the outcome number of mosquito adults.
(TIF)

S7 Fig. Forest plot of comparison: Non-randomised controlled trial on RIDL (release of insects with dominant lethality) *Aedes aegypti* vs. control for the outcome ovitrap positivity.
(TIF)

Author Contributions

Conceived and designed the experiments: LRB PJM. Performed the experiments: LRB. Analyzed the data: LRB SD. Wrote the paper: LRB SD PJM.

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Review

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Evidence-based vector control? Improving the quality of vector control trials

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Vector-borne diseases (VBDs) such as malaria, dengue, and leishmaniasis cause a high level of morbidity and mortality. Although vector control tools can play a major role in controlling and eliminating these diseases, in many cases the evidence base for assessing the efficacy of vector control interventions is limited or not available. Studies assessing the efficacy of vector control interventions are often poorly conducted, which limits the return on investment of research funding. Here we outline the principal design features of Phase III vector control field studies, highlight major failings and strengths of published studies, and provide guidance on improving the design and conduct of vector control studies. We hope that this critical assessment will increase the impetus for more carefully considered and rigorous design of vector control studies.

Evidence-based policy making on vector control

VBDs such as malaria, dengue, and leishmaniasis are responsible for considerable morbidity and mortality and fall disproportionately on the poorest communities in the developing world [1–4]. One of the key methods by which VBDs can be controlled and eliminated is through vector control [5–10]; for example, long-lasting insecticidal nets (LLINs) for malaria or indoor residual spraying (IRS) for Chagas disease.

Development of vector control interventions follows a multistage process [11] (Figure 1). First, a draft target product profile should be generated. This document guides the development process by outlining the features and performance targets of the intended vector control tool. The next step is demonstrating the proof of concept by conducting Phase I studies (laboratory assays to determine the mode of action) and Phase II (semi-field and small-scale field) studies, which generally have entomological end points. Large-scale Phase III field studies (efficacy studies) (see Glossary) are then conducted, which measure the efficacy of the vector control tool against epidemiological outcomes when implemented under optimal conditions.

Based on the results of Phase III trials, the World Health Organization (WHO) will make recommendations for pilot implementation. These Phase IV studies will assess the effectiveness of the vector control tool when it is delivered and used operationally (i.e., under 'real-world' conditions), as well as collecting information on feasibility, distribution mechanisms, acceptability, economics, and safety. Information gathered from the Phase III and IV studies will enable the WHO to draw up policy recommendations and, in parallel, member states will develop country-level policy.

Evidence-based policy making on vector control tools is now regarded as essential and is adopted by the WHO [12,13] (Box 1). The quality of evidence on vector control interventions from epidemiological trials or systematic reviews needs to be rated before recommendations and policy can be formulated. Since 2008, the WHO has adopted the Grading of Recommendations Assessment, Development, and Evaluation (GRADE) methodology for evaluating evidence for policy and guideline recommendations [14,15]. According to the GRADE methodology, an initial rating is given based on the study design. Randomised controlled trials (RCTs) are rated as high-quality evidence and non-RCTs as low quality. Studies are then up- or downgraded based on several factors. RCTs can be downgraded depending on risk of bias, inconsistency, indirectness, imprecision, or publication bias. Non-RCTs can be upgraded based on the effect size observed, dose response, or plausible residual confounding. The final score generated can range from high (i.e., further research is very unlikely to change our confidence in the estimate of

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Glossary (adapted from [24,88–90], http://www.cochrane-handbook.org)

Allocation concealment: refers to keeping the investigator unaware of to which group (i.e., treatment or control) an individual or cluster is assigned. Selection bias can be introduced if the investigator or participant can foresee the assignment (e.g., use of alternation or rotation, assignment envelopes not sealed, not opaque, or not sequentially numbered).

Attrition bias: refers to systematic differences between those individuals or communities that withdraw from the study or those that are lost to follow up versus those that continue in the study.

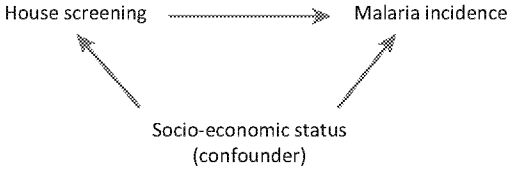
Blinding: a procedure used in trials in which participants/investigators/outcome assessors do not know to which group the individual or cluster has been assigned. Single blind refers to either the participant or investigator/outcome assessor being blinded, while double blind refers to both the participant and the investigator/outcome assessor being blinded.

Case-control study: a study in which a group of people with the disease of interest (cases) and a group of people without the disease (controls), but representing the population from which the cases originated, are identified. The prevalence of the exposure of interest (e.g., use of protective intervention) is compared between these two groups.

Cluster randomisation: a study in which clusters are randomly assigned to either control or intervention groups. Clusters can be geographical areas (e.g., sectors of a large city), communities (e.g., villages), administrative units (e.g., district, region), institutions (e.g., schools), health facilities, or households.

Cohort study: a study in which two groups of disease-free people are identified – exposed (using a protective intervention) and unexposed (not using a protective intervention). The groups are then followed over a period of time for the outcome of interest (usually disease or infection). In this study type, the people are not allocated to the intervention of interest.

Confounding bias: according to Porta, 'confounding occurs when all or part of the apparent association between the exposure and the outcome is in fact accounted for by other variables that affect the outcome and are not themselves affected by exposure' [90]. A variable that is on the causal pathway between the exposure and the outcome is not a confounder. Confounding bias refers to 'bias of the estimated effect of an exposure on an outcome due to the presence of common causes of the exposure and the outcome' according to Porta [90]. This is a common type of bias in observational studies and nonrandomised trials. For example, in an observational study of the association between house screening and malaria incidence, the relationship is likely to be confounded by socioeconomic status since people in superior houses that use screening are likely to be of higher socioeconomic status, who may, for example, have greater access to other protective measures against malaria such as LLINs.



Control group: a group of study participants that receive no intervention, a placebo, or the standard of care depending on the study design and thereby serve as a comparison group when the intervention results are evaluated.

Controlled before-and-after study (CBA): also known as a pre–post study. A study in which observations are made before and after implementation of an intervention in both the intervention group and a control group that does not receive the intervention.

Courtesy bias: a tendency for study participants to give favourable answers out of courtesy to the investigator (e.g., incorrect reporting of high compliance with an intervention).

Crossover study: a study in which individuals/clusters receive the intervention or control for a period of time before switching to receive control or intervention. There is usually a washout period in-between to avoid carry-over effects.

Cross-sectional study: in an analytical cross-sectional study, information is collected at one point in time on the prevalence of the outcome of interest (e.g., disease, infection) and the exposure (e.g., use of a protective intervention).

Detection bias: refers to systematic differences between groups in how outcomes are determined. For example, clinicians assessing patients may be more or less likely to diagnose a particular disease if they know that a person received a protective intervention in the study. Detection bias can be reduced by ensuring that investigators and outcome assessors are not aware of which intervention participants have received.

Effectiveness study: these studies estimate the effect of an intervention under pragmatic or 'real-life' conditions (e.g., intervention delivery under routine conditions so that the relevance of the findings for policy and practice is maximised).

Effect size: the magnitude of difference between treatment and control groups (e.g., risk or rate ratio, percentage reduction in prevalence).

Efficacy trial: these studies estimate the effect of an intervention under highly controlled conditions (e.g., maximal coverage of the target population and adherence to the intervention).

Experimental study: a study design in which we allocate exposure to study subjects and observe the outcome.

Interrupted time series (ITS): a study in which the outcome is measured on several occasions both before and following introduction of an intervention (the 'interruption'). This allows us to see whether an intervention has had an impact greater than any underlying trend in the data. This study design may or may not include a parallel control group.

Observational study: a study design in which we observe the effect of the exposure on the study subjects but no role is played in assigning the exposure to the participants.

Performance bias: according to Porta, refers to 'systematic differences in the care provided to members of the different study groups other than the intervention under investigation' [90]. For example, if participants know they are in the control group of a trial of repellents, they may be more likely to use other forms of vector control, such as protective clothing. Alternatively, health-care providers may care for patients differently if they are aware of which study group they are in. Performance bias can be reduced by blinding to ensure that participants, health-care providers, and researchers are unaware of which intervention participants have received, although this is not always possible.

Randomisation: individuals or clusters are allocated to intervention and control using a random method. Randomisation comprises two interrelated steps, sequence generation and allocation concealment (not to be confused with blinding).

Randomised controlled trial (RCT): individuals or clusters (cluster-randomised controlled trial) are randomly allocated to receive either intervention or control. Intervention and control groups are then followed up for the outcome of interest.

Recall bias: refers to systematic differences between groups in the recall of information regarding exposures. It is a particular problem in case-control studies where surveys are used to gather information on past exposures.

Selection bias: refers to 'bias in the estimated association or effect of an exposure on an outcome that arises from the procedures used to select individuals into the study or the analysis', according to Porta [90]. Often, selection bias refers to systematic differences between the characteristics of the study population and those of other populations and thus there is a lack of generalisability. Nonrandomised studies are particularly susceptible to selection bias, although randomised studies can suffer from selection bias if randomisation procedures are not followed correctly. Selection bias can also be introduced into observational studies. For example, in case-control studies selection bias is introduced if cases are selected that are not representative of all cases within the population or controls are selected that are not representative of the population that produced the cases.

Sequence generation: a method of generating an allocation sequence. The method can be nonrandom (e.g., odd or even date of birth, investigator preference) or random (e.g., random number generator, drawing lots, coin tossing).

Step–wedge design: studies in which the intervention is rolled out to clusters in a staged fashion. At the end of the study, all clusters will have received the intervention. The order in which clusters receive the intervention is usually determined at random.

Stratification/stratified randomisation: a technique used to ensure that equal numbers of individuals or clusters with a characteristic thought to affect response to the vector control intervention (e.g., baseline incidence) will be allocated to each study arm. Multiple clusters are grouped to form strata based on a characteristic (e.g., low versus high incidence of disease) and clusters are randomly allocated within the strata such that equal numbers are assigned to intervention and control. Within each strata more than one cluster is assigned to an arm.

Systematic review: according to Porta, a systematic review is 'a review of the scientific evidence which applies strategies that limit bias in the assembly, critical appraisal, and synthesis of all relevant studies on the specific topic' [90]. The Cochrane Collaboration produces 'gold-standard' systematic reviews that are conducted in a highly rigorous fashion.

Time series: a study in which the outcome is measured on several occasions following the introduction of an intervention. This study design generally has a parallel control group, but may not be randomised.

effect) to very low (i.e., very uncertain about the estimate of effect).

While vector control interventions are the backbone of many disease control programmes, the evidence supporting their use remains weak. Based on our experience systematically reviewing the literature [16–20], we have identified repeated problems with vector control studies. To advance evidence-based policy making, the quality of evidence on vector control interventions – specifically the

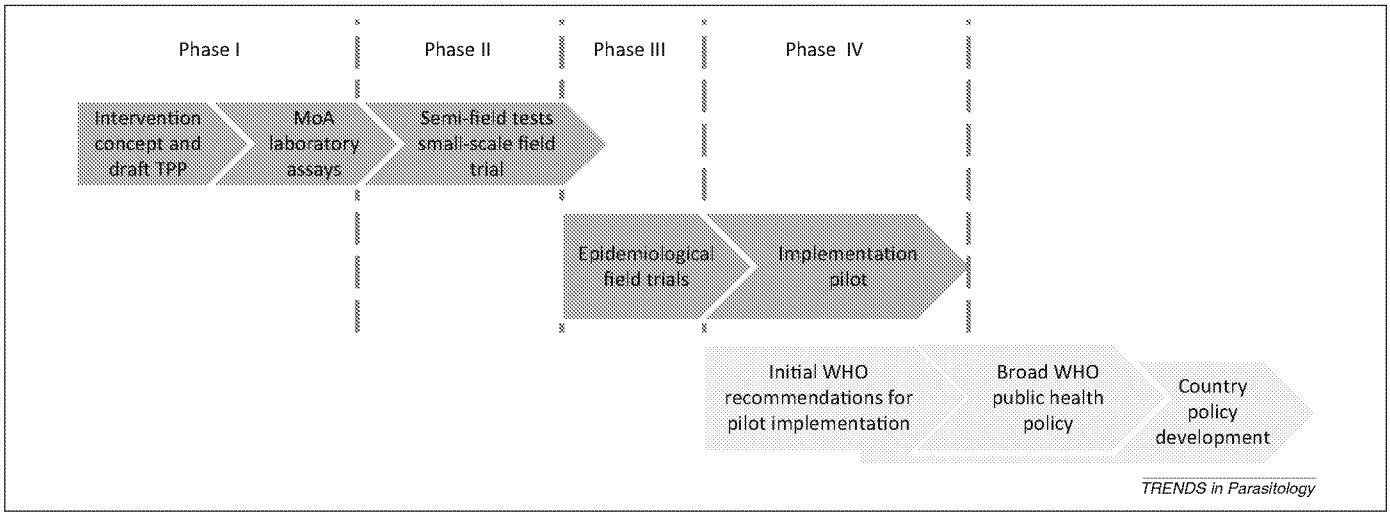


Figure 1. Stages in development of a new vector control product. The first step in the development of a new vector control product is to define the target product profile (TPP), including target efficacy characteristics, safety, and cost. Laboratory assays are then conducted to establish the mode of action (MoA) of the product followed by Phase II studies (semi-field and small-scale field trials) to determine the efficacy of the product against entomological outcomes. Phase III field trials to assess the efficacy of the intervention against epidemiological outcomes are then conducted and, based on the results of these trials, the World Health Organization (WHO; http://www.who.int/neglected_diseases/vector_ecology/VCAG_resources/en/) will make recommendations for pilot implementation. Phase IV pilot implementation studies assess the effectiveness of the vector control tool when it is used under 'real-world' conditions and collect information on feasibility, distribution mechanisms, acceptability, cost, cost-effectiveness, and safety. On the basis of Phase III and Phase IV studies, the WHO develops broad WHO public health policy on which many member states base country-level policy. Adapted from [11].

design, conduct, analysis, and reporting of vector control studies – needs to be improved. The problem of waste in research has recently been highlighted in a series in *The Lancet* that calls for better design, conduct, analysis, and reporting of studies [21,22]. Here we respond to *The Lancet*'s demand to reduce waste in research by highlighting the essence of good study design for evaluating the efficacy of vector control interventions. Given the importance of study design and risk of bias to the GRADE assessment of quality of evidence, we first provide a primer on study designs and bias to illustrate the hierarchy of experimental designs for estimating intervention efficacy. Second, we review common failings of vector control efficacy studies in terms of their design and conduct and suggest how these studies can be improved.

Box 1. Current policy-making process at the WHO [13] [Vector Control Advisory Group (VCAG) Operational Procedures (http://www.who.int/neglected_diseases/vector_ecology/VCAG_resources/en/)]

The WHO has in its mandate to set, communicate, and promote the adoption of evidence-based norms, standards, policies, and guidelines. It is important that this process is streamlined because many countries rely on WHO recommendations to develop their own policy. Two WHO departments are responsible for the main vector-borne diseases: the Global Malaria Programme (GMP) and the Department of Control of Neglected Tropical Diseases (NTDs), which covers other VBDs including dengue, Chagas disease, leishmaniasis, human African trypanosomiasis, onchocerciasis, and lymphatic filariasis. Both departments have advisory committees that provide independent strategic advice and technical input for the development of WHO policy recommendations [i.e., the Malaria Policy Advisory Committee (MPAC) and the Strategic and Technical Advisory Group (STAG) of the Department of Control of NTDs]. These advisory committees are guided by standing technical expert groups and/or *ad hoc* evidence review groups that are responsible for reviewing studies on specific issues and making evidence-based recommendations. New or innovative vector control paradigms are assessed by the WHO VCAG. This group was established in 2013 to guide the development of new vector control paradigms that have the potential for use as public health interventions. The VCAG can be consulted by innovators for advice on developing early-stage vector control paradigms and assesses proof of concept of new vector control technologies. Once satisfied that proof of principle has been established and field trials have satisfactorily demonstrated the efficacy of new forms of vector control, the VCAG makes recommendations to the MPAC and STAG on whether WHO guidelines should be formulated regarding the deployment of the new paradigm for public health use.

General considerations on study designs for vector control studies

The methodological quality of study designs varies such that some are better than others in being able to answer the question 'Does the intervention work?' or 'Does this intervention work better than that intervention?' [23]. In Figure 2 we provide a hierarchy of study designs for evaluating the efficacy of vector control interventions – ranking studies as level 1, 2a, or 2b according to their methodological quality – and list nonrecommended studies. We accept that different study types may be better for answering other questions, such as the acceptability of the intervention [23].

RCTs are generally considered the 'gold-standard' study design for evaluating the efficacy of a protective intervention since they have a low risk of selection bias [24] (http://www.cochrane-handbook.org), which is arguably the most important type of bias in experimental studies. Such is the importance of randomisation that we consider RCTs as level 1 evidence. If the number of randomisation units is sufficiently large, randomisation will ensure that, in a two-armed study, any factors that may affect an outcome are similar in the two arms [24]. Even if one randomises, it is good practice to check that the baseline characteristics of the groups are similar to verify whether the randomisation was successful [25]. If there is no random allocation of intervention and control communities, potential bias can

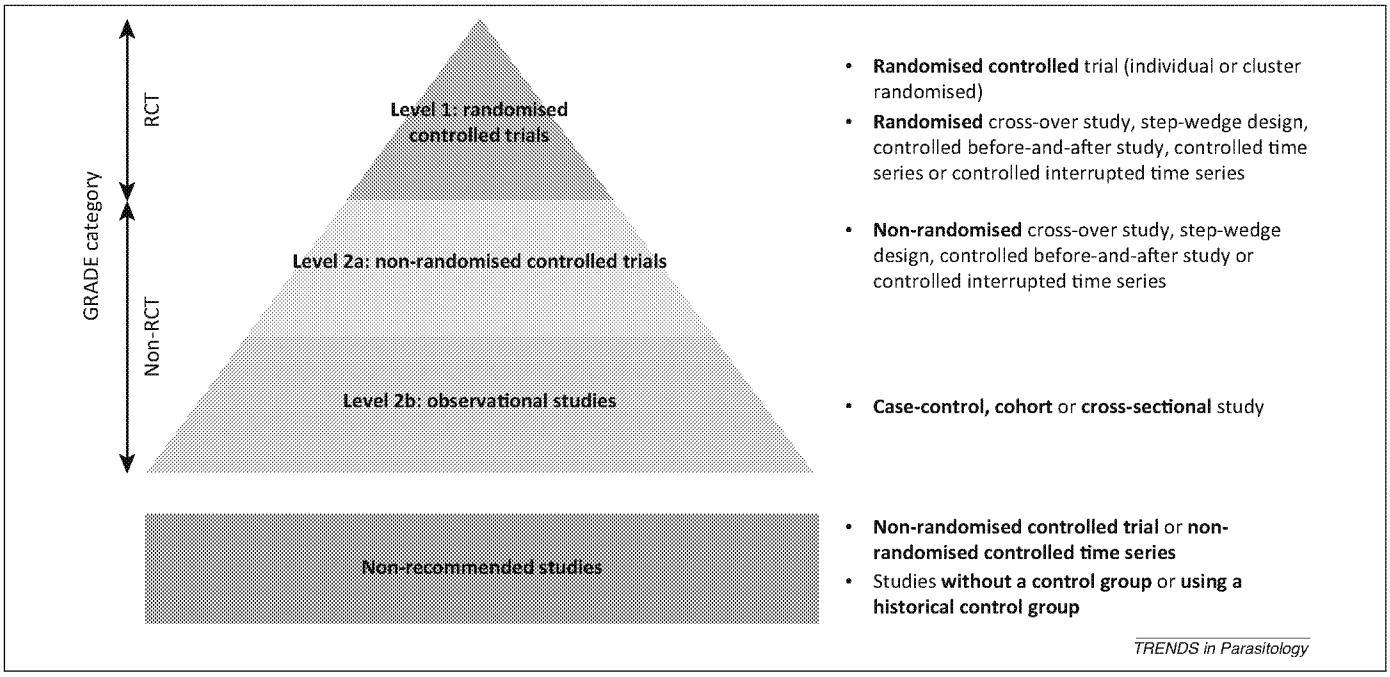


Figure 2. Hierarchy of study designs for assessing the efficacy of vector control interventions. Study designs for assessing the efficacy of vector control interventions can be ranked according to their methodological quality. Randomised controlled trials (RCTs) (level 1) are the 'gold-standard' study design for evaluating the efficacy of vector control interventions. Randomisation reduces the risk of selection bias by ensuring that control and intervention groups are similar to each other. Level 1 studies include cluster or individually randomised controlled trials as well as randomised crossover, randomised step–wedge, randomised controlled before-and-after, randomised controlled time series, and randomised controlled interrupted time series studies. Nonrandomised trials (including nonrandomised crossover, nonrandomised step–wedge, nonrandomised controlled before-and-after, and nonrandomised controlled interrupted time series studies) are at a higher risk of bias and so are ranked lower (level 2a). Observational studies, such as case-control, cohort, and cross-sectional studies (level 2b), provide weaker evidence on the efficacy of protective interventions than experimental designs since they can be subject to bias due to confounding factors and flaws in measuring exposures and outcomes. Nonrandomised controlled trials, nonrandomised controlled time series designs, and studies without a control group or using a noncontemporaneous control group are not recommended. Adapted from an Australian Government National Health and Medical Research Council 2009 document on additional levels of evidence and grades for recommendations for developers of guidelines (http://www.nhmrc.gov.au/guidelines-publications/information-guideline-developers/resources-guideline-developers) and the Oxford Centre for Evidence-Based Medicine 2011 Levels of Evidence (http://www.cebm.net/index.aspx?o=5653). GRADE (Grading of Recommendations Assessment, Development, and Evaluation) levels defined as in [14].

be reduced by adjusting for pre-intervention differences in the two groups using multivariate analysis (e.g., [26]). There is, however, no guarantee that this will fully control for confounders that may be unknown or unmeasured.

In vector control studies, the intervention is often allocated to a group of individuals known as a cluster (e.g., district, village, household) rather than at the individual level. There are several reasons why cluster allocation is common [24]. First, many vector control tools are, by their nature, applied to groups of people or communities. For example, spatial repellent may be allocated to a household or an environmental sanitation intervention against dengue may be allocated at the community level. Second, cluster allocation can help reduce contamination between study arms that might occur if individuals within the same community received different interventions; for example, sharing of insect repellent with family members within the same household or village. Last, cluster allocation means that we are able to assess the community-level effect of the intervention. For example, mass killing of mosquitoes coming into contact with LLINs can reduce transmission so that indirect protection is provided to individuals not using LLINs.

There are numerous other study design types, including controlled before-and-after (CBA) studies, controlled time series, controlled interrupted time series (ITS), crossover studies, and step–wedge designs (Figure 3), that may be more suitable for evaluating the efficacy of some vector control tools. For example, time series or ITS are probably more appropriate for studies of human African trypanosomiasis in which vectors are highly mobile and control efforts need to be implemented over large areas [27]. Step–wedge studies involve rolling out the intervention to clusters in a staged fashion. This design is often used where logistical, practical, or financial constraints make the staged roll out of the intervention desirable. We classify randomised CBA, randomised time series, randomised ITS, and randomised step–wedge studies as level 1 and nonrandomised CBA, nonrandomised ITS, and nonrandomised step–wedge studies as level 2a. We do not recommend the use of nonrandomised controlled trials or nonrandomised time series designs since selection bias is likely to be high and there are no pre-intervention data to assess the comparability of groups.

Observational studies such as case-control, cohort, or cross-sectional studies (Figure 4) have been used to generate evidence of the efficacy of vector control interventions. However, these designs provide weaker evidence than experimental (randomised) designs since they can be subject to bias (e.g., recall bias, detection bias, confounding). For this reason we have ranked these studies as level 2b.

We also do not recommend the use of studies without a control group or those using a noncontemporaneous control group. This is because longitudinal changes, such as rainfall, may impact epidemiological outcomes and can exaggerate or mask an intervention effect.

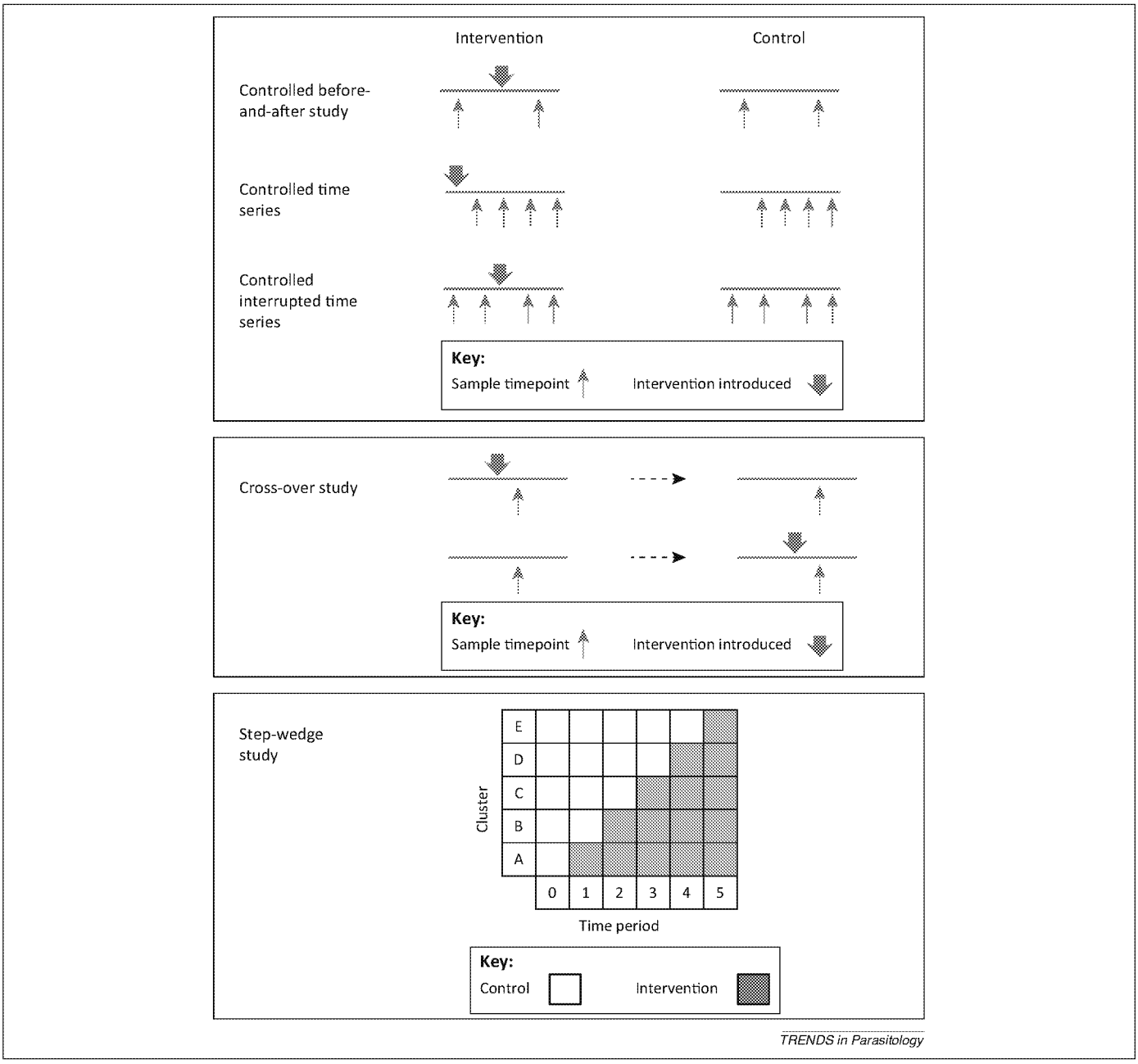


Figure 3. Schematic illustrating design of controlled before-and-after, controlled time series, controlled interrupted time series, crossover, and step–wedge studies. Controlled before-and-after studies involve collecting data on outcome measures before and after implementation of the intervention in the intervention group and at the same time points in the control group. In controlled time series studies, data on outcome measures are collected at several time points once the intervention has been implemented in the intervention group and at the same time points in the control group. Controlled interrupted time series studies involve collecting data on outcome measures at several time points before and after implementation of the intervention in the intervention group and at the same time points in the control group. In crossover studies, two groups are allocated (usually randomly) to control or intervention and outcome measures are assessed once the intervention has been implemented. Following a suitable washout period, the intervention and control are switched around and outcome measures are assessed again. In a step–wedge study, the intervention is rolled out randomly to clusters in a staged fashion so that by the end of the study all clusters will have received the intervention. Adapted from [32].

Common failings of vector control studies and recommendations

Here we describe common problems with the design of vector control studies illustrated with examples and make recommendations for improvements.

Implementation and adherence to the intervention

In efficacy trials, vector control interventions should ideally be implemented in an optimal manner with attention to quality control, high coverage, and user compliance. Unless these parameters are measured, it is impossible to know whether an observed lack of effect is due to low quality, coverage, and/or compliance or lack of efficacy of the vector control method.

Quality control checks should be put in place to ensure that vector control interventions such as IRS are implemented optimally (e.g., correct application of insecticides, coverage of all assigned structures). This can be achieved through accurate record keeping, random spot checks, and supervision [28,29].

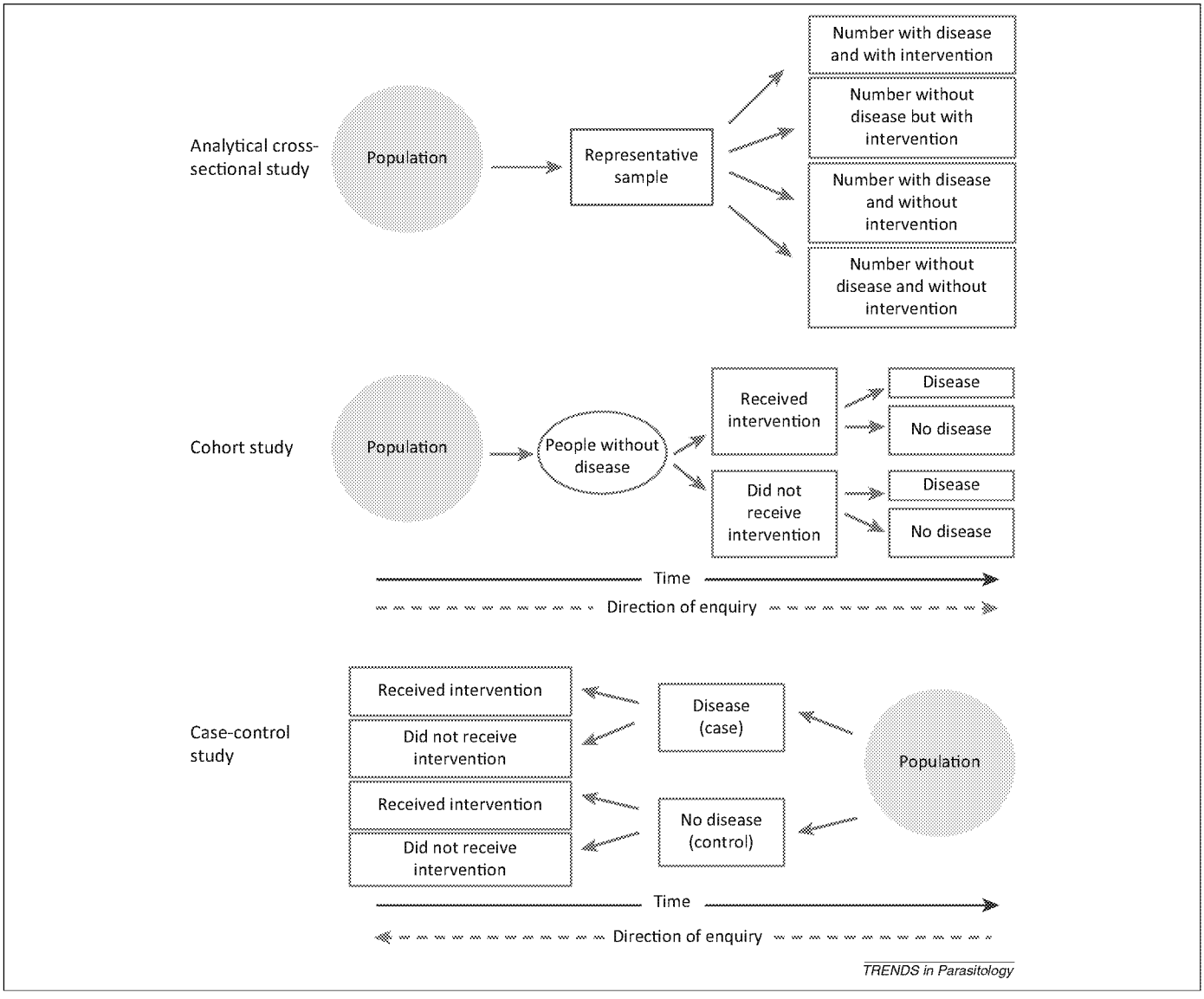


Figure 4. Schematic illustrating design of observational studies for vector control interventions. In an analytical cross-sectional study, a cross-sectional survey is taken from a representative sample of a population. The survey gathers information on outcomes (e.g., disease/infection) and exposure to the intervention from individuals at the same time so the sample can be split into four groups: those with the disease who were exposed to the intervention; those without the disease who were exposed to the intervention; those with the disease who were not exposed to the intervention; and those without the disease who were not exposed to the intervention. In a cohort study, a sample of the population is chosen that is free of disease. Individuals without the disease are split into two groups (those exposed to the intervention and those not exposed to the intervention) and are followed up over time to determine how many develop the disease or infection. In a case-control study, individuals are selected on the basis of their disease or infection status. A group of individuals with the disease or infection (cases) and a group without the disease/infection (controls) are selected. The prevalence of exposure to the intervention is then compared between the cases and controls.

Adherence to the intervention being tested is very important. Efficacy studies usually employ specific techniques (e.g., behaviour change communication) to encourage optimal uptake and use of the intervention where user compliance is required (e.g., [30]). Adherence to the intervention should be measured while taking into account that there is also the potential for introduction of bias here (e.g., courtesy bias). In some cases, innovative methods need to be identified to assess compliance. For example, a RCT of topical repellents against malaria measured compliance through self-reporting of use, the proportion of lotion used estimated from returned bottles, and 'sniff checks' whereby trial staff visited villages at dusk and smelled the arms of participants to check whether lotion had been applied [31].

Choice and measurement of outcome measures

Epidemiological outcomes are necessary to demonstrate the efficacy of the intervention in protecting human populations and to ensure the relevance of these studies to public health. To date, however, many Phase III studies often focus exclusively on entomological outcomes, which are generally useful only for demonstrating proof of concept or as a secondary outcome in support of an epidemiological primary outcome. For example, a Cochrane systematic review on larvivorous fish for malaria control did not identify any studies with epidemiological outcomes [32]. The best epidemiological measure is the incidence of clinical disease or disease-specific mortality, but for some diseases, such as dengue, seroincidence (seroconversion in sequential blood draws) and the prevalence of infection in

single blood draws, including age-specific antibody prevalence, can be good substitutes [30,33]. Studies should use WHO-recommended case definitions with parasitological diagnosis, or serological or molecular verification [34–38] to allow comparison of data between studies. Outcome measures such as self-reported malaria as used by Kroeger *et al.* in a study of repellent soap [39] are unreliable.

Detection bias can be reduced by blinding outcome assessors to the identity of study arms and by the use of objective and well-standardised epidemiological and entomological outcomes. The latter should particularly be used in nonblinded studies.

Entomological data should be collected in a standardised fashion across study arms and sites and over time. Ideally these sampling tools should be automated (e.g., CDC light trap, sticky trap, other trap or target) and not depend on the ability of the fieldworker to collect specimens (e.g., human landing catches, aspiration of resting adults, larval surveys). Several other techniques can help avoid introduction of bias in the measurement of entomological outcomes, including separating the field teams that are implementing and monitoring the intervention (e.g., [40]).

Entomological end points are not always good predictors of epidemiological outcomes. For example, a RCT of LLINs for visceral leishmaniasis reported a reduction in sandfly density in homes but did not show any effect on infection in study participants [41,42]. The authors postulated that transmission was also occurring outside the home and so, although there was a reduction in indoor sandfly density, this did not reduce disease burden. Where possible, it is preferable to use entomological outcomes that relate to disease transmission, such as entomological inoculation rate, rather than measures that do not, such as vector density.

Traditional indicators of immature *Aedes* abundance, such as house index (percentage of houses with larvae and/or pupae), are a poor indication of adult production [43] (http://apps.who.int/iris/handle/10665/68575). Pupal demographic surveys (pupae per person/area index) or measurement of adult vector density are likely to be more appropriate for assessing transmission risk and directing control operations [44,45] (https://extranet.who.int/iris/restricted/handle/10665/69354, http://apps.who.int/iris/handle/10665/68575). However, both measures are far more labour intensive than larval surveys and so may not be feasible for routine monitoring of vector populations [35]. Because, unlike infections caused by protozoa and nematodes, dengue virus infection results in sterilising immunity, pupal and adult surveys are not consistently informative about dengue risk without an understanding of the underlying susceptibility of the human population to dengue virus [46–48].

Avoiding performance bias

Blinding of trial participants, health-care providers, and researchers to the intervention received by participants can reduce performance bias. However, blinding of vector control studies is often impossible. For example, it was not possible to blind study participants in a RCT assessing the efficacy of house screening versus no house screening against malaria [49]. The study found that children living in screened homes were less likely to use bed nets than children residing in homes that were unscreened, which may reflect a belief among householders that screening was a substitute for bed nets. However, the effect of performance bias in this study was minimised because bed net use was carefully recorded and its effect could be adjusted for in the statistical analysis. Alternatively, an originally blinded study may become unblinded during the study. For example, some participants in a RCT of topical repellents became aware that the placebo lotion they were allocated was not providing protection against mosquito bites, which led to the withdrawal of all households in one village [31]. This kind of participant response can lead to introduction of attrition bias.

Selection of sites for entomological monitoring

Sampling sites for entomological surveys are often chosen purposely based on where high vector densities are likely; for example, sites close to suspected larval habitats or houses with unplastered walls or wood construction for *Triatoma* surveys [50–52]. However, this does not measure average community exposure to infection and there is potential for the introduction of sampling bias if sites are not selected in a consistent way across intervention and control arms. We therefore recommend that sampling sites for entomological surveys be selected randomly. It is also possible to separate the sampling frame into strata and sample from each stratum independently, if there is likely to be substantial variation within subpopulations. For example, Joshi *et al.* stratified dwellings into two groups (houses occupied by humans alone and houses occupied by humans and animals) before using simple random sampling to select dwellings in which to measure sandfly density [53].

Contamination or spillover effects

Contamination or spillover effects between different study arms due to the movement of vectors [54,55] or humans between clusters can make interpretation of study findings difficult. Spillover that has a conservative effect (i.e., it biases results towards the null) can occur through one of two routes. First, community-level effects of the intervention can reduce the transmission intensity in neighbouring control clusters, as occurred in a study of insecticide-treated water-jar covers and window curtains against dengue in Mexico and Venezuela [56]. Second, movement of people between intervention and control clusters (and vice versa) is also able to dilute the intervention effect because a person's risk of infection is proportional to the amount of time he or she spends in versus out of the treatment area. If the protective effect of an intervention or the sample size of the study is sufficiently large, a positive result can still be demonstrated in a superiority trial, albeit with reduced intervention effect. However, a negative finding of 'no difference' in such a trial is harder to interpret and a critical question arises. Is the lack of effect due to spillover or due to the absence of efficacy of the new intervention?

A more serious problem arises if the spillover effect is anticonservative, because it exaggerates the difference in outcomes between the intervention and control arms of the

study. For example, topical repellents or house structural changes that have no killing effect on mosquitoes may divert vectors to nonusers in the control arm of the study, putting them at higher risk of infection than they would otherwise have been [57,58].

Hayes and Moulton [24] outline several methods for reducing contamination, including ensuring clusters are well separated, using a buffer zone so there is no common boundary between intervention and control clusters, as shown in a larval source management study conducted in Tanzania [59], or a 'fried-egg' design where the intervention and control are administered throughout the cluster but only the central portion is used for outcome measurement [60]. When designing these types of studies it is, therefore, important to have an estimate of how far the vector is likely to fly in seeking a blood meal or a breeding site. Georeferences of cases that constitute the outcome measure should be recorded to show whether there were edge effects due to contamination. This technique has been used to estimate the size of area-wide effects in studies of LLINs for malaria control [61]. Unintended consequences of topical repellents can be avoided by randomising only a relatively low proportion of individuals or households in a village to receive the intervention [31,62,63]. Tackling the problem of human movement in dengue studies is more difficult because *Aedes aegypti* feeds during the day when people are engaged in their daily activities. Potential strategies to avoid this would be to use larger cluster areas or monitor epidemiological outcomes in a sentinel cohort that is less mobile (e.g., young children) [64]. Even if these steps are taken it is a good idea to collect travel histories from study participants, particularly if the intervention is located in a household. In this way, participants can be excluded from the per-protocol study analysis if they have travelled for significant periods of time and, therefore, spent a relatively brief time being exposed to the intervention (e.g., [65]).

Contamination can also be a problem in crossover trials if the washout period is insufficient. While crossover trials may be suitable where the washout period is short (e.g., larvicide with a short half-life [66]), they should be used with caution where interventions are persistent (e.g., DDT, habitat manipulation).

Need for sample size calculations

Sample size calculations are performed before conducting a study to quantify the power that the study has to show an effect of the intervention and thereby answer the study question (Box 2). The effect of a small sample size is on the standard error of the outcome measure; that is, it will lead to large confidence intervals around the estimated effect and hence poor precision. The sample size needs to be large enough to ensure that the probability of a type II error is reasonably small, generally 10% (= 90% power) or 20% (= 80% power). Sample size calculations should be performed for all study outcomes, whether epidemiological or entomological. We identified several studies that did not report conducting sample size calculations for epidemiological and/or entomological (e.g., [67–71]) outcomes, including several studies that failed to show an effect of the intervention [72,73], indicating that the lack of an effect may simply be due to the study being underpowered. Parameters required for sample size calculations such as the prevalence or incidence of the outcome in the control group or the coefficient of variation may not be readily available [30], although the former can be estimated from a survey conducted before the study's start if it is not known.

Vector control trials generally use a cluster design. Since outcomes measured in individuals or sampling sites within the same cluster are likely to be more similar than those between clusters, the sample size calculation needs to take this into account and a larger sample size is required than when a nonclustered design is used (Box 2). Hayes and Moulton recommend the use of six clusters per arm as an absolute minimum and it is generally better for cluster-randomised trials to have a higher number of smaller clusters than fewer large clusters [24]. We identified a large number of published vector control trials that used two villages [74,75] or two areas [76,77], one in which the intervention was introduced and the other acting as a control. This is a poor design because the use of only two clusters means that the intervention effect is completely confounded by study site and effectively constitutes a sample size of one [78,79].

Box 2. Power and sample size calculations [91–93]

When conducting a study there are two hypotheses that need to be considered: the null hypothesis (there is no difference between the two interventions) and the alternative hypothesis (there is a difference between the two interventions or, more commonly for superiority trials, the novel intervention is more protective than standard practice). When testing a hypothesis there are two types of error possible:

- Type I error, or α. We reject the null hypothesis incorrectly (i.e., there is no effect but we report that there is).
- Type II error, or β. We incorrectly do not reject the null hypothesis (i.e., there is an effect but we fail to detect it).

Several factors need to be considered when calculating sample sizes.

- The prevalence or incidence of the outcome in the control group.
- The expected effect size of the new intervention. It is important to be clear about what is the smallest size of effect we deem to be relevant from a public health or clinical perspective; for example, a study assessing the effect of house screening against exposure to malaria vectors established at the beginning of the trial that full screening or screened ceilings would be recommended if they reduced house entry by malaria mosquitoes by at least 50% [49].
- Significance level (*P* value). This represents the probability of a type I error; generally 0.05 is used, which means that we have a 5% probability of a type I error.
- Power. The power of a study is the probability of not committing a type II error, or 1 − β (e.g., if we have a 20% probability of a type II error, the power is 80%).

Many vector control trials use a clustered design. For cluster-randomised trials, two additional factors need to be taken into account:

- Average cluster size.
- The coefficient of variation, *k*, which measures the level of between-cluster variation of the outcome.

This is important because outcomes measured in individuals or sampling sites within the same cluster are likely to be correlated. A large value of *k* implies substantial between-cluster variation in the outcome, which makes it harder to show an intervention effect unless the sample size is increased.

It is recommended to consult an experienced statistician to assist with sample size calculations, particularly for cluster-randomised trials.

Table 1. Minimum recommended follow-up periods by study type[a]

Study design	Preintervention	Postintervention
Randomised controlled trial	Desirable to check baseline characteristics of study population At least one transmission season for entomological data if sampling sites are nonrandomly selected	At least one transmission season (two seasons is desirable)
Controlled before-and-after study	At least one transmission season, especially if entomological sampling sites are nonrandomly selected	At least one transmission season
Randomised controlled time series	Not applicable	Two or more transmission seasons
Interrupted time series	Two or more transmission seasons	Two or more transmission seasons
Crossover study	At least one transmission season before crossover (and washout) and one transmission season after	

[a]Transmission season may be shorter than a 1-year period or a whole year if transmission is perennial.

Deciding on the duration of the follow-up period

Insufficient periods of follow up plague many vector control trials. For example, a RCT of topical repellents against malaria in Ethiopia conducted two malaria prevalence follow-up surveys 1 month and 2 months after the baseline survey [80]. This study is unlikely to give a true picture of the efficacy of the repellent since compliance with the repellent would probably remain high during this short time period but decline over a longer time period. It is also worth noting that *Plasmodium falciparum* infections last on average 1 year [81,82], although they can persist for up to a decade or longer [83], and it takes several years for this indicator to re-equilibrate fully following a reduction of transmission [84,85].

For entomological outcomes, follow-up periods need to be sufficiently long and repeat measurements need to be taken to gain a picture of transmission in the area (e.g., [86,87]). This is because there is likely to be large variation in vector density between sampling sites and across different sampling periods (night to night, week to week, or over a transmission season) due to environmental factors such as rainfall. Designs in which entomological sampling is conducted once during the follow-up period are less likely to give reliable results due to inherent variability in vector populations even if the number of sampling units is high. Longer periods of follow up with repeat measurements can be used to assess whether the effect of an intervention is waning (e.g., IRS with a short-lasting insecticide) and to determine how often the intervention needs to be replaced or reapplied.

We recommend that minimum pre- and postintervention follow-up periods be used for epidemiological and entomological data collection, the duration of which differs depending on the study design chosen and the context of pathogen transmission (Table 1).

Concluding remarks

We have identified common problems with vector control studies and provide suggestions on how these can be improved. We also illustrate that some study designs are methodologically stronger than others. While hierarchies based on study design are somewhat controversial (http://www.alliance4usefulevidence.org/publication/what-counts-as-good-evidence-february-2013/), we believe they remain useful in addressing the evidence for what interventions work, particularly when combined with a broader evaluation of the quality of the evidence as offered by GRADE [14,15]. More specifically, the GRADE rating of evidence takes into account numerous factors in addition to study design [14,15]. This means, for example, that a poorly conducted RCT with a high risk of bias does not necessarily constitute better evidence than a sound observational study with a large effect size.

We suggest that there are several reasons why many vector control studies have historically been designed and conducted in a less-than-optimal fashion. First, a lack of resources may have limited the extent to which entomologists could conduct large-scale, well-designed studies. This may help explain the large number of two-village comparison studies and studies without epidemiological outcomes. The impact of shortfalls in resources is exacerbated by issues associated with implementing environmental interventions on a large scale and the urgent need for VBD control. Second, medical entomologists have traditionally not been taught epidemiology or have not worked in an integrated fashion with epidemiologists. It is necessary to upgrade this aspect of the skill set of medical entomologists, to include epidemiology in medical entomology course curricula, and for epidemiologists to partner with entomologists in conducting intervention assessments.

New vector control tools are urgently needed to reduce the burden of VBDs. In highlighting key problems with the design and conduct of vector control tools and suggesting remedies we hope that this manuscript will provide an impetus for upgrading the evidence base on vector control interventions. The present lack of rigorous, evidence-based vector-borne intervention assessments is an obstacle to innovation in disease reduction. It also wastes a considerable amount of money, time, and energy. Improving the quality of future vector control trials will not only save valuable resources but will also expedite the process of achieving recommendation from the WHO for the roll out of effective new interventions.

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Findings from the Technical Workshop to Create a Safe, Effective and Integrated Strategy for the Control and Elimination of *Aedes aegypti* from Puerto Rico

Hosted by the Puerto Rico Brain Trust for Tropical Disease Research and Prevention, an initiative of the Puerto Rico Trust for Science, Technology and Research.

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This Technical Workshop can be summarized by five points:

- The *Aedes aegypti* mosquito is the root cause of Dengue Fever, Chikungunya and Zika transmission.
- Experts in mosquito control and public health convened in Puerto Rico to determine the feasibility of eliminating *Ae. aegypti* infestation from the island.
- There was broad agreement that the elimination of *Ae. aegypti* and the diseases it carries is complex but feasible through Integrated Vector Management using physical, chemical and biological interventions on an area-wide basis.
- Public engagement and authorization is critical to program success including building partnerships with Puerto Rican communities and stakeholders.
- Strong leadership, dedicated program management, and autonomy of the entity charged with the execution of this campaign are essential to successful elimination.

EXECUTIVE SUMMARY: Findings from the Technical Workshop to Create a Safe, Effective and Integrated Strategy for Control and Elimination of the *Aedes aegypti* Vector from Puerto Rico.

The *Aedes aegypti* mosquito is the root cause of Dengue Fever, Chikungunya and now Zika transmission, which has brought a new level of urgency to eliminate this mosquito. On May 24, 2016 a workshop of 47 technical experts in mosquito control and public health was hosted by The Puerto Rico Trust for Science, Technology and Research. Experts focused on answering the question, "Can the *Ae. aegypti* mosquito be controlled or eliminated in Puerto Rico thereby eliminating the disease? If so, how?" Our conclusion was that it is possible to eliminate *Ae. aegypti* from Puerto Rico with a well-managed vector control program. While elimination is possible, we also concluded that the *Ae. aegypti* population could first be reduced below the threshold of disease transmission and, from that vantage point, the decision to fully eliminate this mosquito can be taken based on a cost-benefit analysis. Area-wide elimination of *Ae. aegypti* has been done before and, in our opinion, it can be done again. Our work included an assessment of the mosquito control methodologies available today, the current state of mosquito-borne disease in Puerto Rico and a review of historical elimination programs that have successfully eliminated disease-carrying mosquitoes. The full report outlines: 1) the interventions that, when properly integrated, have a high probability of controlling or eliminating *Ae. aegypti*; 2) the near-, mid- and long-term introduction of each vector control tool; and 3) the infrastructure and leadership that must be in place to ensure effective execution of the project.

Safe and effective tools to deploy the mosquito control program include physical interventions to eliminate breeding sites, chemical interventions that kill larvae and adult mosquitoes and biological interventions, notably the inundative release of male mosquitoes that cannot productively mate, thereby preventing female mosquitoes from producing the next generation; this method is broadly referred to as the Sterile Insect Technique (SIT). Integration and management across interventions along with data on mosquito populations and geographical disease burden is essential to the success of the program. The first year of a program should be the most intensive. A master plan of how interventions will be staged over time and by geography for maximum effect should be created at the outset. The plan should be widely shared as part of a community engagement strategy. Regulatory issues should be identified with an action plan for safe and expedient oversight by the appropriate agencies. In this first year, physical and chemical interventions will be prominent while biological intervention (SIT) manufacturing facilities are being constructed in Puerto Rico. Elimination of *Ae. aegypti* infestation will focus on highly burdened regions of disease in Puerto Rico, with SIT finishing the job in years two and three. From there, a "rolling front" of elimination will move on to second-tier disease-burdened regions. The decision to eliminate *Ae. aegypti* from the entire island will be taken in year four using cost-benefit analyses based on regional elimination. As elimination is achieved, on-going port-of-entry surveillance and control operations should be maintained to prevent re-infestation.

The interventions proposed were well understood with a high degree of consensus across the experts. We converged on one critical missing piece for implementing these recommendations: to effectively execute any mosquito control program requires a dedicated, autonomous mosquito control organization with the mandate to execute the program. It seems Puerto Rico is missing this basic capacity, which exists throughout the continental US in the Mosquito Control Districts (*e.g.* the Florida Mosquito Control Districts). Therefore, above all, Puerto Rico should establish a Mosquito Control Commission to carry out the *Ae. aegypti* control program and maintain it after the current crisis is solved to ensure that the island never faces this threat to human health and the economy again.

FULL REPORT: Findings from the Technical Workshop to Create a Safe, Effective and Integrated Strategy for Control and Elimination of the *Aedes aegypti* Vector from Puerto Rico.

The *Aedes aegypti* Mosquito: Root Cause of Zika, Dengue and Chikungunya Disease Transmission

The *Aedes aegypti* mosquito vector is the root cause for transmission of several serious infectious diseases including Dengue, Chikungunya, and Zika[1,2]. Dengue has been an endemic health problem in Puerto Rico for decades and globally more than 390 million people are infected yearly[3]. Some estimates place Dengue infections at 3.9 billion worldwide[4,5]. Chikungunya has caused illness, loss of the ability to work and has resulted in long lasting consequences for many Puerto Ricans[6]. The Zika epidemic, with the serious consequences of microcephaly, Guillian-Barré Syndrome and potential long-term neurological disorders, has brought a new level of urgency to eliminate *Ae. aegypti*, the source of transmission[7]. *Ae. aegypti*-transmitted diseases have also brought severe economic consequences to Puerto Rico with cumulative losses in the hundreds of millions of dollars due to associated health costs and cancelation of tourism and sporting events[8].

A Community without *Aedes aegypti* Mosquito Infestation is Free from the Diseases It Transmits

On May 24, 2016 a workshop that brought together 47 technical experts in mosquito control and public health was convened by The Brain Trust for Tropical Disease Research and Prevention, an initiative of The Puerto Rico Trust for Science, Technology and Research. The group explored an integrated vector elimination strategy for *Ae. aegypti* in Puerto Rico. The workshop focused on answering the question, "Can the *Ae. aegypti* mosquito be eliminated thereby eliminating the disease? If so, how?"

Our workshop concluded that it is possible to eliminate *Ae. aegypti* from Puerto Rico with a well-managed vector control program. While elimination (defined as greater than 90% reduction in *Ae. aegypti* populations island-wide) is possible, we also concluded that the *Ae. aegypti* population may be first controlled and, from that vantage point, the decision to eliminate *Ae. aegypti* can be taken based on a cost-benefit analysis[9]. Health objectives are met most effectively by focusing on highly infested areas with the most disease. Area-wide elimination of *Ae. aegypti* has been done before on a scale that far exceeds that of Puerto Rico and, in our opinion, it can be done again.

Our work included an assessment of the mosquito control methodologies available today, the current state of mosquito-borne disease in Puerto Rico and a review of historical elimination programs that have successfully eliminated disease-carrying mosquitoes, for example Fred Soper's elimination program of the African malaria vector *Anopheles arabiensis* from a 54 thousand km^2 area in Brazil in 1939[10] or Oswaldo Cruz's campaign against *Ae. aegypti*[11]. These campaigns eradicated disease in the Americas through the 1950's. However, by the 1970's failure to maintain control measures led to the resurgence of mosquito-borne disease. Much has changed since prior "top down" elimination campaigns; we live in a dynamic, modern and democratic society. Cargo transport has facilitated the spread of *Ae. aegypti* while discarded tires and plastics have aided its breeding[12]. Enforcing the clean-up of mosquito breeding sites on private property today may be more challenging but modern times have also given us powerful tools to fight this mosquito. On balance, it is our conclusion that by combining the traditional and modern tools for mosquito elimination with good leadership, the odds of successful mosquito elimination today are better than ever. This report outlines: 1) the interventions that, when properly integrated, have a high probability of eliminating *Ae. aegypti* and maintaining elimination; 2) the near-, mid- and long-term introduction of each vector control tool; and 3) the infrastructure and leadership that must be in place to ensure effective execution of the project.

Recommended Safe and Effective Physical, Chemical and Biological Strategies

Safe and effective tools for mosquito control can be grouped into three main categories: physical, chemical and biological interventions. Physical interventions include the physical elimination or removal of breeding sites. Chemical interventions are defined as the use of registered insecticides that kill mosquitoes directly. For the purposes of this study, biologically produced larvicides such as *Bti (Bacillus thuringiensis israelensis)*, are classified as chemical interventions because they are operationally deployed identically to chemical larvicides[13]. Biological interventions refer to active living organisms that reduce mosquito populations, including mosquito-eating fish, entomopathogenic fungi, and especially the inundative release of sterile male mosquitoes preventing females from producing the next generation of mosquitoes (SIT). Finally, good intelligence on mosquito prevalence and breeding sources as well as geographical disease burden is essential to establish a baseline and monitor the success of the program.

Recommended Physical interventions emphasized the reduction of *Ae. aegypti* breeding sites (water meters and septic tanks). Physical interventions tend to be labor intensive and require a high degree of public compliance because of the need for resident participation. If well executed, community programs can empower citizens to reduce breeding areas in and around their homes, creating a community that is invested in the solution. It was recommended to engage local communities through outreach programs as well as to train and manage a large team of breeding-source reduction employees to do routine island-wide clean-up of breeding sources. Expert management of this team is recommended to bring efficiency to this labor-intensive activity. Modern, mobile phone-based communication and social media could be employed to facilitate good communication with commonly available technology. Excellent compliance will need to be enforced. However, in the Puerto Rican context, incentives may be more effective than punitive measures. Fines for failure to remove breeding sites from private property must be a strong option, but might not be the first option if incentives work.

Recommended chemical interventions. It was generally recommended to use a combination of safe (EPA approved) and effective larvicides and adulticides. *Ae. aegypti* have become resistant to many adulticides and a database of resistance should be widely disseminated to guide usage. It is important to quickly bring the regulation of insecticides in Puerto Rico in line with the continental United States and eliminate multiple layers of regulation that are peculiar to Puerto Rico[14]. For example, organophosphates and insect growth regulators are insecticides to which local *Ae. aegypti* are still susceptible, but regulatory issues have complicated their use specifically in Puerto Rico. Because *Ae. aegypti* is a day-biting, indoor-resting mosquito, special attention must be paid to insecticide application. Indoor Residual Spraying is most likely to control the *Ae. aegypti* vector and reduce disease if applied by trained personnel that understand where *Ae. aegypti* spends time in the house[15]. There is some controversy on the use of over-the-counter (OTC) products that could be used by consumers. OTC products may help people reduce *Ae. aegypti* in their homes, but there is a possibility of user abuse which puts health, safety and efficacy at risk. Outdoor residual spraying may be practical in areas where outdoor *Ae. aegypti* populations are higher than average or where access to the inside of homes/businesses is difficult. Aerial and ground spraying by truck should be guided by an understanding of how the insecticide will effectively come in contact with the target, *Ae. aegypti* or its breeding sites[16]. One clever way to deliver larvicides to cryptic breeding sites is to use Auto

Dissemination (AD) traps. AD traps destroy distal breeding sites as the mosquitoes visiting the AD trap pick up a larvicide and transport that agent to hidden breeding sites. Since *Ae. aegypti* breeds in places that are hard to reach, the AD trap exploits the mosquito's egg-laying behavior to find and treat hard-to-reach breeding sites[17]. Another emerging tool is luring adult *Ae. aegypti* to traps baited with either attractive toxic sugar baits, or moisture for resting or oviposition. Above all, a trained labor team managed by mosquito control experts is needed for the implementation and close monitoring of an island-wide chemical intervention strategy.

Recommended Biological Interventions. A major tool that has not previously been widely available at scale for mosquito control is the Sterile Insect Technique (SIT)[18]. This method relies on the release of male mosquitoes that effectively find and mate with females but cannot produce viable offspring. Because males do not feed on blood or transmit disease, they can be released safely in large numbers. By inundating the area with these male mosquitoes that cannot bite or productively mate with females, the population can be dramatically reduced or eliminated. Since this intervention relies on mating within the *Ae. aeqypti* species only, there is no collateral damage to other insect species, mammals or birds. Because *Ae. aeqypti* is a non-native invasive species that makes up only ~1% of the mosquito population, there is little if any disruption to the ecosystem upon targeted removal. The United States Department of Agriculture (USDA) presented several success stories of area-wide elimination of agriculturally important insect pests using a combination of chemical insecticides and the Sterile Insect Technique. SIT has been extensively used for over 50 years for the population control of major agricultural insect pests and disease vectors. In these cases, irradiation has been used to sterilize males, which are then released to find females and prevent future offspring[19]. USDA has implemented this method to eliminate insect pests with great success. Screw-worm[20], Pink Bollworm[21] and Medfly[22] are examples of area-wide insect elimination over geographies hundreds of times larger than Puerto Rico. Releasing insects from aircraft has proven cost-effective for covering large areas. Engineers have now developed methods to deliver sterile *Ae. aegypti* from aircraft[23].

Three types of SIT were presented that could be used to control or eliminate *Ae. aeqypti*[24]. First, a genetically engineered *Ae. aegypti* has been developed to produce males with non-viable offspring. Field results were presented from outdoor pilot programs in several countries resulting in >90% reduction of *Ae. aegypti* with this genetically engineered mosquito[25,26]. Second, male mosquitoes may be infected with the bacterial endo-symbiont *Wolbachia* to produce males that cannot productively mate with local females if they do not carry the same bacteria[27]. Promising semi-field results were presented and the first outdoor field trials of these infected male mosquitoes are in progress now. Finally, as demonstrated by the USDA programs, irradiation can be used to create sterile male mosquitoes. For mosquitoes, the combined irradiation and treatment with *Wolbachia* was proposed which allows the release of sterile males while at the same time ensuring that there will be no release of fertile and/or pathogen transmitting females[28,29]. Promising semi-field results were presented and the first outdoor field trials of this combined approach are in progress now.

The experts from this workshop recommend some form of SIT as part of an Integrated Vector Management strategy to eliminate *Ae. aegypti* because of the demonstrated success in area-wide elimination of other pests by USDA. With several attractive SIT options, the right option should be chosen based on the greatest likelihood of safe and effective elimination of *Ae. aegypti* with special attention paid to logistical requirements and operational feasibility.

Monitoring of Ae. aegypti mosquito populations and Ae. aegypti-associated disease

The Centers for Disease Control and Prevention along with Puerto Rico Department of Health currently track *Ae. aegypti*-associated disease regionally across Puerto Rico, including Dengue, Chikungunya and -Zika[30,31]. We recommend supporting this disease monitoring over the course of the *Ae. aegypti* control/elimination program as the ultimate measure of program success. As an immediate indicator of *Ae. aegypti* control, we recommend the trapping and monitoring of the adult *Ae. aegypti* using Autocidal Traps of some form[32]. These traps are used to monitor populations, but can also be used in higher density to significantly reduce mosquito populations. Population data from trapping must be aggregated, processed and presented to track and manage program interventions. The data may also be useful to share vector elimination efforts transparently with policy makers and the public [33]. With a geographical view of the *Ae. aegypti* infestation and disease distribution, the program should first focus on highly burdened areas for elimination, such as Caguas, San Juan and Ponce. Upon successful elimination in these high burden areas, the program may confidently expand to second tier burdened areas (see timeline). Continued monitoring provides feedback on intervention strategies island-wide. Monitoring of strategic areas, such as points of entry, will be indefinite and accompanied by a Quick Reaction Force (QRF) that can be deployed immediately to suppress incursions from ports of entry. Experts propose using the mosquito and disease data in conjunction with demographic data and climate and weather patterns to inform statistical modeling programs developed outside the program in academia [34]. In the future, such models may help prevent or prepare for vector disease outbreaks globally.

Community engagement and participation

Even the most solid interventions can fail for lack of public participation, or understanding of the health objectives or consideration of the community and context of the intervention. In this case, "top-down" interventions are at risk if the community rejects the intervention at the outset or is unable to sustain the intervention over time. A modern program might be neither "top down" nor "bottom up", but seek to hybridize the strengths of each approach[35,36]. While the new tools for mosquito control may be more effective in eliminating *Ae. aegypti*, departures from traditional methods may bring controversy. Concerns about the environment are now featured in public consciousness and should be addressed in the pursuit of public health. In addition, active public engagement and authorization is critical to program success and we recommend building partnerships with communities and other stakeholders[37]. We recommend the use of interviews, focus groups, and town hall meetings to understand attitudes and practices of the communities in which the programs will be executed. Household and school-based awareness programs, communication strategies and media campaigns that reach every part of the community are important. Encouraging community-based vector-control strategies that promote cleaning up breeding sites engage the community in the program while reducing the source of mosquitoes. Strategies that empower communities to contribute to the source reduction may have higher acceptance rates, visibility and impact on vector densities[38].

Timeline for Implementation of Interventions

<u>*Short term – first year*</u>. Experts agreed that the first year of a program should be an intensive period of activity. A master plan of how interventions will be staged over time and by geography for maximum effect should be created at the outset of the program. The plan should be widely shared and gain the support of all stakeholders as part of the engagement plan. Specific community issues should be

recognized up front with an action plan to address each issue. Regulatory issues should be identified in advance and an action plan created for safe and expedient oversight by the appropriate agencies. An over-arching mosquito control authority must be established as a first priority. In this first year, Autocidal Traps will be deployed and a monitoring system will be put into place that interfaces with the current disease monitoring at the Centers for Disease Control and Prevention and the Department of Health. This monitoring system will incorporate other relevant factors, such as climate, rainfall, mosquito dwelling and biting behaviors, characteristics of humans infected, geographical location of traps with regard to housing and other useful data. A baseline *Ae. aegypti* population map should be created paying special attention to areas of urban density and large municipalities where *Ae. aegypti*-associated diseases have been most prevalent. Physical and chemical interventions should be deployed with great vigor focusing particular attention on the training and management of the labor force that will be deployed to clean up breeding sites and deliver insecticides to the targets. Auto Dissemination (AD) traps should be deployed to get at hard-to-reach breeding sites. This first year is critical for the establishment of the SIT program, which may be fully deployed in year two. A call for proposals in the first three months will identify the appropriate SIT intervention and provider(s).

Mid-term – second and third year. In the second year, the interventions that were initiated in year one will be expanded. *Ae. aegypti* population data will become available to evaluate the effectiveness of interventions and the most effective strategies will be reinforced. In the second year, SIT will become available and, where *Ae. aegypti* populations have been knocked down, SIT may be used to regionally eliminate *Ae. aegypti*. Concentrating the SIT intervention to follow physical and chemical interventions in high-disease-burdened municipalities, such as San Juan or Ponce, should visibly drive down disease in this critical second year building momentum to reinforce the program's elimination objectives.

As regional deployment of SIT eliminates the remaining *Ae. aegypti* in high disease-burden areas, the intensity of the physical and chemical interventions may move to the second tier of disease-burdened geographies in year three. This will create a "rolling front" of *Ae. aegypti* elimination with intense physical and chemical interventions on the leading edge and SIT deployed for elimination expanding behind the leading front. Experts recommend maintaining diligent monitoring of *Ae. aegypti* and associated disease in cleared areas so that *Ae. aegypti* does not outflank the program. Continuing public engagement at this time is important as the disease burden begins to wane and the public's priorities may shift. Although not a part of vector control, by year three the possibility of vaccines to address Dengue, Chikungunya and Zika will be more apparent. While a Dengue vaccine is available in several countries, approval by the US FDA is still pending. Vaccines for Zika and Chikungunya are in development and may take more than a decade to be clinically tested and approved.

Long term – year four and five. At this point we should have a view on the "end game" and what regions remain for elimination. With the experience of the previous three years, we can examine the cost-benefit of elimination of *Ae. aegypti* versus control. As we have learned from other SIT programs for agricultural pests, elimination of *Ae. aegypti* with SIT may be less costly than allowing a low-level persistent population that can serve as a reservoir for re-infestation. Alternatively, permanent suppression of mosquito populations in towns and cities may be cheaper than constant high density monitoring and island-wide preventive sterile releases to avert re-infestation in Puerto Rico. A detailed cost-benefit analysis will be needed.

If elimination is chosen, ongoing monitoring of *Ae. aegypti* importation and spot removal is essential, as is done for the medfly SIT program maintained by the USDA in California. Using these programs as a guide the *Ae. aegypti* program in Puerto Rico may employ similar port-of-entry surveillance and island-wide monitoring to detect local mosquito outbreaks[39]. Control measures may include continued preventative release of sterile males, lethal trapping and insecticidal control in areas that are vulnerable for introductions. Finally, if a vaccine for Dengue, Chikungunya and Zika has become available for distribution, we may consider this alternative, assuming a plan to vaccinate is in place, especially for pregnant women.

Management, Infrastructure and Capacity-Building

The interventions proposed were well understood, leading to a higher degree of consensus than expected across the 47 workshop experts. In the end, we converged on one critical missing piece for implementing these recommendations: to effectively execute any mosquito control/elimination program requires a dedicated, autonomous organization with the mandate to execute the program. It seems Puerto Rico is missing this basic capacity, which exists throughout the continental US in the Mosquito Abatement Districts (*e.g.* the California and Florida Mosquito Control Districts)[40,41].

Therefore, above all, Puerto Rico should establish a Mosquito Control Commission to carry out the *Ae. aegypti* control or elimination program and maintain it after the current crisis is solved. To ensure the technical capability required for program activities, we suggest that the Puerto Rico Mosquito Control Commission should be led by a mosquito control professional like those that run mosquito control districts elsewhere in the US with a long-term commitment and funding. This Director must have the authority and budget to execute an ambitious plan, just as Fred Soper had when he eliminated disease-carrying mosquitoes in his day. Because mosquito control is not an activity well suited to a medical or agricultural agency, it is imperative that the commission is independent from existing governmental structures and budgets. A Board, which may include representation from the PR Department of Health, CDC, Department of Agriculture and Natural Resources, for example, could appoint the Director. The Board should be charged solely with the oversight of the Director, with the authority for hiring the Director and holding him/her accountable for results. The Director should have complete autonomy and accountability for the elimination program. This Commission should continue over time so that once *Ae. aegypti* and the mosquito-borne diseases are under control and out of the public consciousness, a preventative program remains. The Zika, Chikungunya and Dengue epidemics occurred for lack of sustained vector control. Constant vigilance is required to prevent the resurgence of mosquito-borne diseases, those we know and those yet to emerge.

In Summary

The resurgence of serious diseases transmitted by the *Aedes aegypti* vector is one of the most urgent and important health issues for Puerto Rico today and its resolution can be found in the elimination of the vector. Vector control and preferably elimination is the only sustainable solution for Puerto Rico to thrive, turning around this crisis in public health as well as what is happening in the loss of tourism, movie production, and sporting events because of mosquito-borne diseases. Failure is not an option. Experts have made recommendations to address Dengue Fever, Chikungunya and Zika in Puerto Rico through vector control, the basis of disease transmission. The proposed first step to implement those recommendations is the formation of the Puerto Rico Mosquito Control Commission.

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Opinion Article

No magic bullet: citizenship and social participation in the control of *Aedes aegypti*

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We were already used to our every summer dengue. A seasonal epidemic that has happened in a relatively predictable repeated manner, since the late 1980s. A condition that is closely related to the seasonality of its urban, domestic, anthropophilic and synanthropic vector.

Due to the absence of effective and large-scale vaccines, and to the lack of a specific treatment against dengue symptoms, the general guideline is to focus on the reduction of the vector density. This was the routine of service managers and health agents.

Most of *Aedes aegypti* breeding sites are located inside households, and in the past years this knowledge initially contributed to place the responsibility on the population. The discussion that followed – "who's the guilty?" – deviated the focus from key issues: sanitation, access to piped water, waste collection, mobilization, disease prevention and health promotion, citizenship etc. The debate has evolved and nowadays the participation of society, in a collective effort, is stimulated.

Everything seemed to go as planned; our problems were being handled (despite sweeping some under the carpet) and nothing was beyond the usual discomfort we were used to manage so far.

Then, a sanitary earthquake takes place in the country, with several shock waves: first, the imminent arrival of chikungunya virus, with an alarming possibility of long-term health compromise of patients;[1] the second was a smooth wave, the emergence of the Zika virus, apparently a disease with mild and short-lived symptoms;[2] the third wave of this sanitary earthquake came with microcephaly in babies, Guillain-Barre syndrome in adults, and also other potential neurological damages. Panic takes over. It mobilizes the population, media, and service managers, reaching other countries and leading to the recognition by the World Health Organization (WHO) that Brazil is passing through an international public health emergency.[3,4]

The world's attention turns to Brazil. The pressure here is felt individually and collectively, inside and outside the academy and the health services environment. People try to create individual solutions to protect themselves and repellents disappear from the shops' shelves; magic recipes for protection and control against the mosquito vector multiply on social networks, and, besides that, charges against the possible culprits, in the best "conspiracy theories" style, arise. After all, it seems that everyone understands a bit about communication and vector control. A significant part of the media, opinion multiplier, engages in mobilizing the society to participate in preventive actions; researchers are called to collaborate and many questions come up.

Some scientists are quick to bring solutions to control the vector, either with already known technologies or with "innovative" or "alternative" approaches. Biomedical solutions include since returning to the emphasis on using insecticides by the method of ultra-low volume (ULV, also known as 'fogging' in many parts of the country) to releasing sterile mosquitoes (produced by genetic modification or by irradiation),[5,6] as attempts to reduce the vector populations. The "Wolbachia-based strategy", a sustainable technology that replaces the populations of *Aedes aegypti* by individuals that are not able to transmit the virus, is also at hand.[7] In other knowledge fields, initiatives that are inspired by the attention given by

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human and social sciences to communities, citizenship and the environment begin to gain relevance, reflecting the maturity of the country in recognizing that diseases transmitted by vectors transcend the sphere of Health Care and require intersectoral actions.[8,9]

In this context, the International Meeting for Implementation of New Alternatives for *Aedes aegypti* Control was held in February 2016 in Brazil under the coordination of the National Program for Dengue Control (*PNCD*), a program that belongs to the Ministry of Health. At that meeting, technologies with the potential to be implemented in the affected cities were evaluated, considering the structural and operational issues of vector control in the country.[10] At that time, some of the methodologies were considered and distributed into the three categories presented below.

1) Recommended approaches for immediate inclusion in *PNCD*

In this category three initiatives were included. All of them have been previously tested in some Brazilian municipalities with satisfactory results and were considered viable to be incorporated into the country's control actions without a significant impact on the program costs or routine:

- The strategy known as eco-bio-social, which focuses on social participation and environmental management in controlling the vector.[11] This approach significantly reduced the vector density in Fortaleza, Ceará State, and is already being applied in two other municipalities, as requested by the Ministry of Health: Goiânia, Goiás State, and Belo Horizonte, Minas Gerais State.
- The risk mapping takes into consideration the spatial heterogeneity in the distribution of infections. This methodology uses relatively simple methods to identify areas that persistently accumulate dengue cases. The proposal, in this context, is to enhance the interventions in those areas.[12,13]
- The spread of larvicide mediated by the mosquitoes themselves, which act as disseminators.[14] The strategy is based on the fact that *A. aegypti* females spread their eggs in many breeding sites, reaching breeding spots that are inaccessible to men, especially in urban environments which are disorganized and are in a vulnerable situation. Mosquito mediated pyriproxyfen dispersion, the larvicide currently used by the *PNCD*, was conducted in the Amazon region by previously trained endemic control agents. A reduction of the vector density of at least ten times was verified.[15]

2) Recommended approaches for inclusion in *PNCD* in special situations

In this category, actions aiming the protection of pregnant women, considered a priority group on the epidemic of Zika virus, were listed. For this group, the recommendation was to include in the routine of the Program the use of window and door screens, with or without insecticides, to keep the mosquitoes away, the distribution of repellents for personal protection and the possibility of intradomiciliar insecticide spraying. However, the impact of these measures on the budget of Brazilian cities, even if applied only to this particular group of people, is an issue that cannot be overlooked. Restricting public resources to be used primarily on protecting public places, such as health facilities and schools, is a possibility to be considered.

3) Potentially promising technologies

This category included strategies that cannot be incorporated immediately in the *PNCD* either because their cost is incompatible with the available public resources, or because the schedule for national-level implementation is not feasible in the short-term, or even because they add important operational issues, such as a deep change in the routine of health agents – which requires time and planning. Wolbachia-infected mosquitoes, sterile mosquitoes and the application of spatial repellents for homes were added here.

Sterile males aim to reduce vector populations. Their sterilization is achieved genetically (transgenic mosquitoes) or through irradiation.[5,6] Females inseminated by sterile males do not generate a viable offspring. Nevertheless, this approach requires a frequent release of massive amounts of sterile males in order to become powerful against vector populations. This is especially relevant in the case of irradiated specimens, who have their survival and viability jeopardized by the process.

The idea behind Wolbachia-infected mosquitoes is different: these mosquitoes are intended to have a dual function, both reducing and replacing the original populations. The presence of these bacteria, precludes or impairs mosquito infection with the dengue and chikungunya virus.[16] There is evidence

that it also happens with Zika virus[17]. The introduction of Wolbachia in *A. aegypti* does not involve genetic engineering. This strategy has a sustainability component missing from the sterile males' technology: Wolbachia-infected females produce more offspring than wild females. Since all eggs derived from those females are already born containing Wolbachia, there is no need for frequent releases of mosquitoes. Besides, wild females inseminated by Wolbachia-infected males cannot produce offspring, causing a reduction in the original population.[18]

There is also a strategy that combines Wolbachia-infected males and irradiation. By using this procedure, already performed with *Aedes albopictus,* it is not necessary, before releasing females in the field, to separate them from males in the laboratory – one of the most expensive stages of the technique. In this case, the sterilization of males happens due to the presence of Wolbachia and the irradiation is used to sterilize females of this lineage. Because these females are more susceptible to radiation, there is little compromise in the viability of males.[19]

It is worth noting that, for both Wolbachia-infected mosquitoes and sterile mosquitoes, the local population partnership is an essential factor. Both methods require strong engagement of communities, since they are based on the release of mosquitoes, a task that is the opposite to the common sense of vector control. This situation reveals an additional evidence of the complexity of this issue and shows that, even if the solution was merely technological, the biomedical technology could not dispense other technologies neither the theoretical-methodological framework typical of human and social sciences, especially information, education and communication.[9]

In addition, a question remains: what is the risk of moving away from the central problem if we give priority essentially to the technical and welfare aspects of vector control? Appropriate medicines are only possible when the diagnostic is correct. Experiences from other countries, and even from some places and situations in Brazil, show that the relation among different government sectors, added to the participation of non-government sectors and the general civil society, are at the basis of a successful control of dengue epidemics.[9] Yet, the question remains: how to support it?

This overwhelming epidemic of Zika virus is an extreme situation, with no precedents, certainly the biggest health emergency in which all living Brazilians have been through. The population is insecure, and this can lead to panic. In whatever perspective we look at, this is a unique opportunity to rethink our assumptions. From an essentially mercantilist point of view, this situation can be an excellent business opportunity. For some academic sectors, it can constitute a great opportunity to gain visibility and curriculum prestige. But it can also be an opportunity for each person to assume his social responsibility and leave his comfort zone, both individually and collectively.

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In2Care® Dissemination Unit

Easy to use
Robust and user-friendly design that does not need a power source.

Novel
Multi-impact tool with new bioactives - using mosquitoes to kill their own offspring

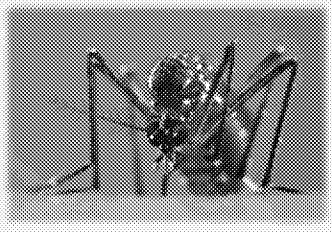
Effective
Lab and field data show that units can effectively control *Aedes* mosquitoes.

A novel tool to combat *Aedes* mosquitoes that transmit Dengue, Chikungunya and Zika virus

Dengue, Chikungunya and Zika virus are rapidly spreading mosquito-borne viral diseases. They are difficult to diagnose and treat, and mosquito control is the only option to stop transmission.

Aedes mosquitoes are difficult to control as they lay their eggs in very small breeding sites and have become resistant to chemical insecticides. The In2Care® Dissemination Unit attracts and kills *Aedes* females with novel sustainable ingredients that target both mosquito larvae and adults. It is the first to exploit the concept of 'auto-dissemination', resulting in an effective kill of mosquito larvae in breeding sites surrounding the unit.

In2Care® Dissemination Units can be placed both in- and outdoors at a recommended density of 1/400 m^2 (10 units per acre) and be maintained every 4-6 weeks using refill sachets. The product lends itself perfectly for use in vector control programs, particularly in hotspot/problem areas, and by pest control companies that offer mosquito control services to resorts, hotels, etc. These user-friendly units can also be used by the general public and enable effective vector control via community participation.



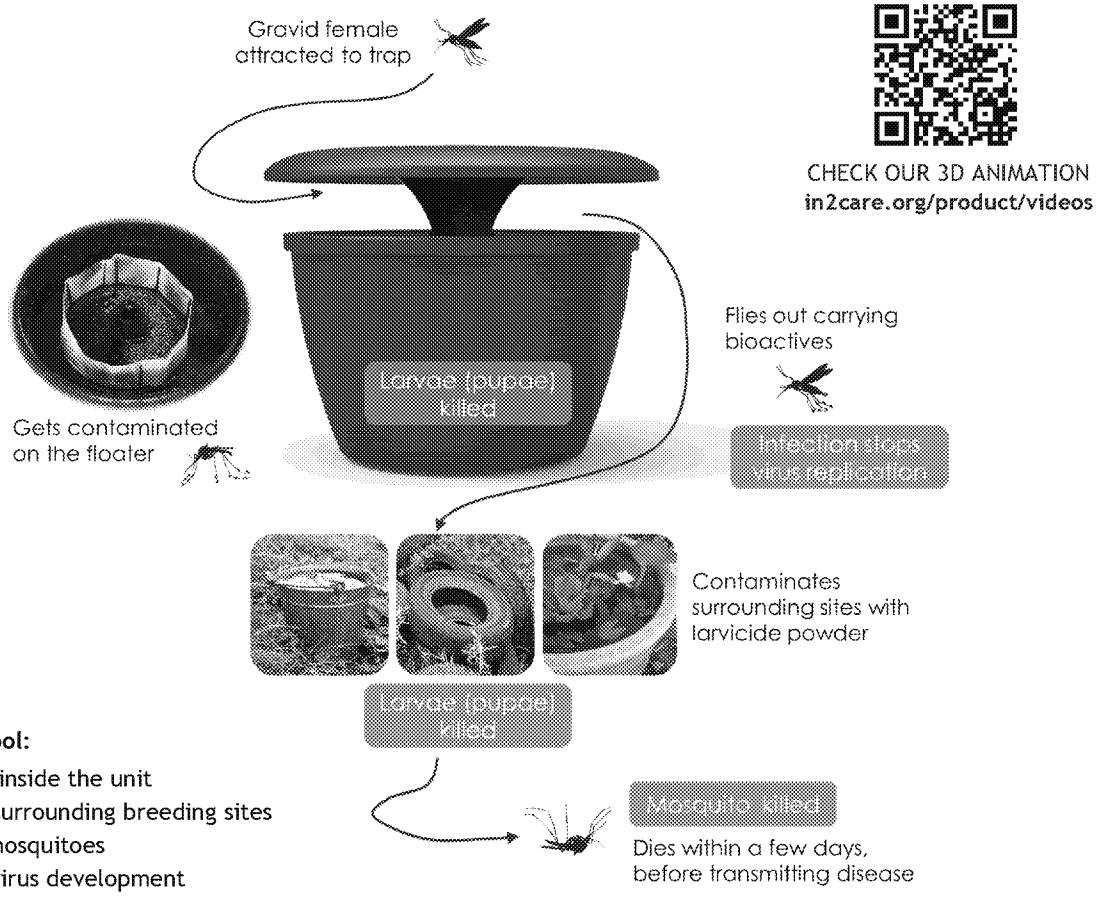
Exploiting mosquito behaviour

Aedes aegypti mosquitoes originate from Africa, but have spread worldwide rapidly and can transmit Dengue, Chikungunya and Zika virus to humans. They are attracted to small container-like breeding sites and have a unique egg-laying behaviour; distributing their eggs over several breeding sites to minimise risks for their offspring.

In2Care® Dissemination Units exploit this behaviour by contaminating the female mosquito body and using her to spread larvicide to multiple breeding sites around the unit. Via this "auto-dissemination" method the unit can kill virtually all mosquito larvae in its surroundings before these become biting adults.

How does it work?

The In2Care® Dissemination Unit is made of durable plastic and uses water with an odour lure to attract egg-laying *Aedes* mosquitoes. Once inside, mosquitoes contact the specially treated gauze near the water surface and get contaminated with a larvicide and a fungus. We exploit the fact that *Aedes* like to divide their eggs over multiple sites; by letting them fly out of the unit whilst carrying larvicide on their legs. They transport the larvicide and contaminate several breeding sites around the unit. In this way, we can kill larvae in small and hard to find breeding sources. The mosquito also gets infected with an insect-specific fungus that can block Dengue virus replication and kills her before she can spread disease.



A multi-impact tool:

- ✓ Kills all larvae inside the unit
- ✓ Kills larvae in surrounding breeding sites
- ✓ Kills exposed mosquitoes
- ✓ Stops Dengue virus development

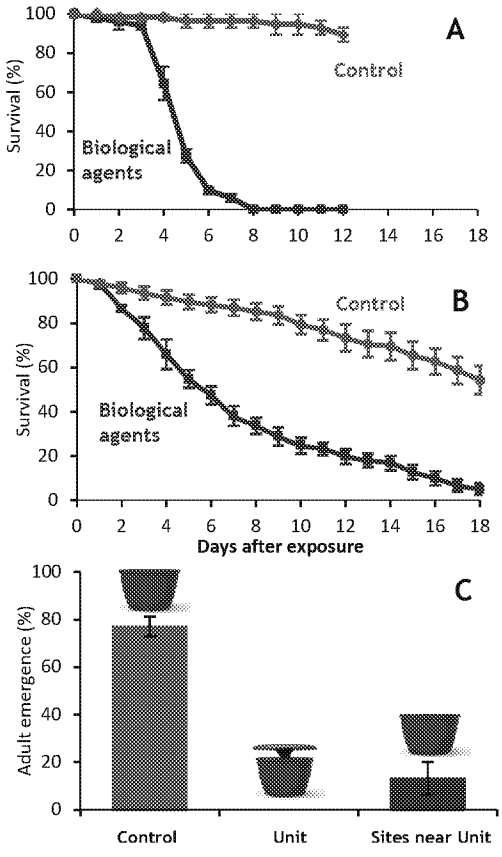
An environmentally friendly solution

Insecticide resistance has become a major problem in countries infested by *Aedes* mosquitoes. Area-wide chemical fogging is still being used but is showing limited efficacy and major impacts on non-target organisms. This necessitates a switch to more sustainable, environmentally friendly vector control. The In2Care® Dissemination Unit is the first device to use a biological control agent to kill mosquitoes. It deploys an US-EPA-approved fungus that kills the mosquito several days after infection and has been shown to be able to block Dengue virus replication inside the insect. The second active is an US-EPA approved larvicide that can even be used in drinking water and has not shown any issues with resistance. Both bioactives have short half-lives and are classified as low risk for non-target organisms.

In2Care® Dissemination Units deploy a small dose of bioactives in an enclosed point-source environment that is specifically attractive to mosquitoes. Only tiny amounts of larvicide will get spread to other breeding sites (mostly small man-made containers), which is enough to kill mosquito larvae because 10 ppb still works well, but is not enough to cause risk for non-target organisms like fish or mammals. In this way, our units offer an effective mosquito control option without drastic use of chemicals in the entire environment.

Published & Field validated Results

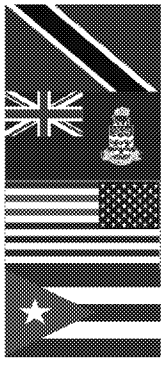
In2Care® Dissemination Units were developed with *Aedes* mosquitoes collected in the Caribbean. Scientific lab validations showed that half the mosquitoes survive for 4 days after gauze exposure (graph A). In large cage tests, whereby mosquitoes were free to visit the unit or 4 other sources when they like, the killing impact took longer (graph B). This does, however, allow contaminated mosquitoes to disseminate larvicide to other sites before they die. This induced a massive reduction in adult mosquitoes produced in breeding sites near the unit; after one day release only 1 in 10 larvae survives to adulthood (graph C). In the unit itself, 100% of the larvae die, mostly in the L_4 or pupal stage. More results can be found in our peer-reviewed publication: **www.parasitesandvectors.com/content/7/1/200.**



The units deploy a very special type of gauze developed by In2Care. It holds bioactives via electrostatic binding forces, which enables a high dose transfer when mosquitoes make contact. The improved bioavailability and resistance-breaking potential of this netting has been published in the prestigious *Proceedings of the National Academy of Sciences USA* (*PNAS*): **http://www.pnas.org/content/112/39/12081.full.pdf.**

Field validations

A field trial executed in 2015 by the Insect Vector Control Division of the Ministry of Health of Trinidad & Tobago demonstrated active auto-dissemination and larval control, and a sustained decline in mosquito densities. Trinidad MoH is now using units in selected problem areas (public schools & hospitals). A scientific field test with 200 In2Care® Dissemination Units executed by the Mosquito Research & Control Unit of the Cayman Islands also showed active larvicide dispersal, effective larval control and reductions in the adult *Aedes* mosquito population. A US-EPA approved semi-field study by the Manatee County Mosquito Abatement District in Florida confirmed unit impacts on larvae and adults of local strains of *Aedes aegypti* and *albopictus* under ambient climate conditions. The Puerto Rico CDC is currently (2017) undertaking a large-scale randomized clustered trial in 10 residential areas (17.5 acres each) to quantify the impact on adult *Aedes* populations.



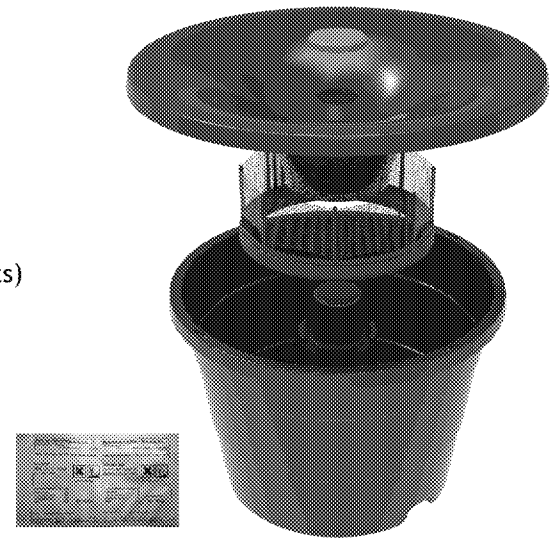
How to use

We recommend placement where mosquitoes are likely to breed: in shaded, vegetated places near habitation. In high risk areas we recommend 1 unit per 400m^2 (10 units per acre). We offer support for appropriate risk mapping of your area and unit density calculations. Unit maintenance (topping up with water) is recommended at regular intervals depending on climate and monitoring demands, and reactivation with a fresh refill sachet is recommended every 4-6 weeks.

Mosquitoes are not trapped but are contaminated. Because of its slow-killing action, you will see live larvae present in the units, but these will die before they pupate into adults. When deployed properly in a large enough area, In2Care® Dissemination Units will effectively reduce the numbers of *Aedes* mosquitoes and the risk of infection with Dengue or Zika virus. Optimal impacts are achieved when removing as many other breeding sources as possible. For non-isolated sites we recommend additional barrier treatments. Effects will become most noticeable after the first 2 weeks of deployment because the next mosquito generation is affected.

The In2Care® Dissemination Unit includes:

- Durable 5L water reservoir
- Lid with click-on mechanism
- Floater (to carry the gauze strip)
- Green time indicator cap (servicing reminder)
- Optional fixation tools
- Refill sachets (gauze, bioactives & attractant tablets) for reactivation every 4-6 weeks



We can provide:

- ❖ Customized deployment support
- ❖ Unit servicing and monitoring support tools
- ❖ Field trial protocols
- ❖ Registration dossiers for product registrations

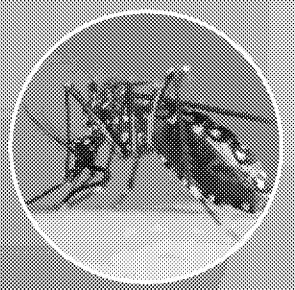
In2Care® Dissemination Units have been registered and are being sold in >25 countries in the Americas by our authorized distributor Univar Environmental Sciences. Our units have been approved for professional use in US states with confirmed local transmission of Zika virus by a Section 18 Emergency Exemption of the US-EPA pending full registration. In Asia, our distributor Ensystex is currently registering the product in >10 countries, and in the Pacific we collaborate with AustralAsian Biosecurity for unit distribution.

For more information on registration, local distributors and sales options, please contact us via customer.support@in2care.org

About In2Care

In2Care BV is a private limited company registered and based in the Netherlands. The core expertise of our team of medical entomologists and product developers lies in the translation of scientific knowledge into novel and user-friendly insect control products. In2Care has in-house R&D capacity including mosquito rearings, and collaborates with renowned scientific institutes to validate the efficacy of our innovations. We have filed study protocols available and can be consulted for advice and customized vector control approaches. We go beyond product development to deliver novel, sustainable, affordable and user-friendly solutions to combat mosquitoes that transmit some of the worst infectious diseases in the world.

Because we are into care



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Novel,
Sustainable,
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Parasites & Vectors

RESEARCH Open Access

Development and evaluation of a novel contamination device that targets multiple life-stages of *Aedes aegypti*

Janneke Snetselaar, Rob Andriessen, Remco A Suer, Anne J Osinga, Bart GJ Knols and Marit Farenhorst*

Abstract

Background: The increasing global threat of Dengue demands new and easily applicable vector control methods. Ovitraps provide a low-tech and inexpensive means to combat Dengue vectors. Here we describe the development and optimization process of a novel contamination device that targets multiple life-stages of the *Aedes aegypti* mosquito. Special focus is directed to the diverse array of control agents deployed in this trap, covering adulticidal, larvicidal and autodissemination impacts.

Methods: Different trap prototypes and their parts are described, including a floater to contaminate alighting gravid mosquitoes. The attractiveness of the trap, different odor lures and floater design were studied using fluorescent powder adhering to mosquito legs and via choice tests. We demonstrate the mosquitocidal impacts of the control agents: a combination of the larvicide pyriproxyfen and the adulticidal fungus *Beauveria bassiana*. The impact of pyriproxyfen was determined in free-flight dissemination experiments. The effect on larval development inside the trap and in surrounding breeding sites was measured, as well as survival impacts on recaptured adults.

Results: The developmental process resulted in a design that consists of a black 3 Liter water-filled container with a ring-shaped floater supporting vertically placed gauze dusted with the control agents. On average, 90% of the mosquitoes in the fluorescence experiments made contact with the gauze on the floater. Studies on attractants indicated that a yeast-containing tablet was the most attractive odor lure. Furthermore, the fungus *Beauveria bassiana* was able to significantly increase mortality of the free-flying adults compared to controls. Dissemination of pyriproxyfen led to >90% larval mortality in alternative breeding sites and 100% larval mortality in the trap itself, against a control mortality of around 5%.

Conclusion: This ovitrap is a promising new tool in the battle against Dengue. It has proven to be attractive to *Aedes aegypti* mosquitoes and effective in contaminating these with *Beauveria bassiana*. Furthermore, we show that the larvicide pyriproxyfen is successfully disseminated to breeding sites close to the trap. Its low production and operating costs enable large scale deployment in Dengue-affected locations.

Keywords: *Aedes aegypti*, Dengue, Ovitraps, Vector control, *Beauveria bassiana*, Pyriproxyfen

Background

Globally, 2.5 billion people are at risk of becoming infected with Dengue fever [1], a mosquito-borne disease for which there is no specific medication or vaccine. With over 390 million cases annually [2], Dengue is currently the fastest spreading infectious disease in the tropics. Costs to contain the disease are huge and put severe pressure on (health) budgets of affected countries. Without drugs or a vaccine, control of mosquitoes that transmit the virus remains the sole option to control the disease. Contemporary mosquito control focuses primarily on larval source management in the form of breeding site removal or larviciding and adult control through fogging with insecticides [3].

The main vector of Dengue is the yellow fever mosquito *Aedes aegypti* (L.), a diurnal species that displays skip-oviposition behavior (i.e. lays small numbers of eggs in multiple sites [4]) and prefers man-made containers as oviposition sites [5]. These sites are often small and

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difficult to locate, which makes effective larviciding difficult. The preference of *Aedes* mosquitoes for container-like breeding sites provides the opportunity to control gravid mosquitoes using ovitraps. An ovitrap basically consists of a black or dark colored container filled with water with one or several attractants to lure mosquitoes. Egg-laying female mosquitoes are attracted to the trap by the water [6], visual cues [7], natural odors (mostly from plant infusions) [8-11], conspecifics [12], or synthetic odors [5,13-15]. Ovitraps have an advantage over other traps (for host-seeking mosquitoes) because they do not require a power source or additional carbon dioxide and are not dependent on trap operator's skill and motivation.

Over the years, various ovitraps have been developed and tested against *Aedes* mosquitoes. Originally, ovitraps were designed as 'egg dump' devices [6], killing all larvae hatching inside the trap. However, since *Aedes* females show skip-oviposition behavior this targets only a minor proportion of the lifetime reproductive output by females. Novel ovitraps were therefore designed to also target the adult mosquito. These traps include designs such as the 'sticky' trap [7,16,17] or 'double-sticky' trap [8] in which gravid mosquitoes are captured using glue, or lethal ovitraps [18-20] in which mosquitoes are exposed to insecticides. A major disadvantage of these lethal ovitraps is the fact that insecticides deployed in such traps have shown reduced efficacy due to widespread insecticide resistance in *Aedes* populations [21].

There are promising alternative mosquito control agents that have been proposed for use in ovitraps, notably autodissemination agents, which are larvicidal compounds that are dispersed to breeding sites by contaminated adult female mosquitoes. Pyriproxyfen is a WHO-recommended juvenile hormone analogue that targets mosquito larvae at the pupal development stage and can be effective in extremely low concentrations (<1 ppb) [22]. It is already being deployed as a mosquito larvicide and is approved for use in drinking water in low concentrations. Experiments have shown that female mosquitoes can acquire pyriproxyfen crystals when landing on a treated surface and deposit these in breeding sites they subsequently visit [23,24], hence killing their offspring and other larvae already present in those breeding sites at the time when these pupate. Because *Aedes* mosquitoes are skip-ovipositors, pyriproxyfen can be used as an autodissemination agent for 'mosquito-driven larval control'; utilizing the gravid female to disperse the larvicide and contaminate multiple breeding sites in the vicinity [25,26].

Field studies with pyriproxyfen have shown good potential for this new type of vector control [26]. Considering that the contaminated mosquito loses the pyriproxyfen crystals from her legs over time [23], it would be advantageous to deploy this agent in such way that the timeframe between pick up and transfer is as short as possible by contaminating gravid females that lay their first batch of eggs. This would be possible via an ovitrap that contaminates the adult mosquito with pyriproxyfen and allows her to leave the trap afterwards. Considering that *Aedes* mosquitoes typically only need a short time-frame to (skip) oviposit, the addition of a slow-killing adulticide to target the contaminated adult would increase the control impact of such a device. Slow-killing biopesticides, such as entomopathogenic fungi, would be suitable candidates for this purpose. Spores of the fungus *Beauveria bassiana* have been shown to effectively infect mosquitoes upon contact by penetrating the insect cuticle and growing into the haemocoel [27]. This infection reduces the mosquito's vectorial capacity [28,29], inhibits Dengue virus replication inside the mosquito [30] and eventually kills the mosquito. An additional benefit of this fungus is that it is highly virulent to insecticide-resistant mosquitoes [27,31] and even has the potential to augment the efficacy of chemical insecticides [27,32]. The relatively slow kill and pre-lethal impacts of *B. bassiana* can prevent Dengue transmission and at the same time enable effective dissemination of pyriproxyfen by contaminated mosquitoes to surrounding breeding sites.

Whereas contemporary ovitraps have shown good potential in reducing the number of *Ae. aegypti* in an area when deployed in sufficiently high numbers [5], they are mainly used for scientific and monitoring purposes and not commonly deployed as a standard *Aedes* control tool. This opens the opportunity for a trap that can be manufactured on a large scale for the pest control market.

Here we describe the development of a new type of ovitrap, a multi-impact contamination device for *Aedes* mosquitoes. Our aim was to create a user-friendly control device that does not rely on electricity or chemical insecticides. We show the steps taken to design a trap that is attractive to egg-laying *Ae. aegypti* and meets requirements for large-scale manufacturing. Experiments were performed to optimize device attractiveness, including tests with several odor lures to augment attraction to *Aedes* mosquitoes. We show how the device design and the deployment of a new type of gauze enables effective contamination of ovipositing *Aedes* females. In the second part of this paper we demonstrate the potential adulticidal, autodissemination and larvicidal impacts of the agents deployed in the trap. We report for the first time the combination of the control agents *B. bassiana* and pyriproxyfen. Experiments were set up to demonstrate the impact of this mixture, including measurements of lethal impacts on contaminated adults, larvicidal impacts inside the trap and larvicidal impacts in surrounding breeding sites.

Methods

Mosquito rearing

Experiments were conducted using laboratory reared *Ae. aegypti* mosquitoes. This colony originates from adults collected in the Caribbean (Aruba) in 2011. Mosquitoes were reared at a temperature of 27(±1)°C and a relative humidity of 65(±5)%. Mosquitoes had an artificial light-dark cycle of 12/12 h (L:D). Mosquito larvae were reared on tap water and fed daily on Tetramin® water tablets for bottom dwelling fish (Melle, Germany). Adult mosquitoes were kept in 30×30×30 cm gauze Bugdorm® cages and had *ad libitum* access to a 6% glucose solution on filter paper. Mosquitoes were fed on human blood twice a week, either through direct feeding on the arm of a volunteer or on membranes.

Trap validation tests utilized gravid females (7-8 days old) at the time they were ready to lay eggs. Prior to the experiments, blood-fed females were selected manually with a mouth aspirator and placed in a container with access to a 6% glucose solution on cotton wool. These females were then kept for 4 days to become gravid, before being used in experiments.

Evaluation of oviposition attractants

To augment the attractiveness of the trap, several odor lures for gravid *Ae. aegypti* were evaluated (Table 1). We tested a commercially available synthetic mosquito odor lure tablet (AtrAedes), which contains odors identified from volatile grass infusions (*Panicum maximum*, Jacq.) and has been used in other oviposition traps designed to lure gravid *Aedes aegypti* mosquitoes [5,13,14]. Teabags, organic water (from a local ditch) and oak leaf (*Quercus* spp.) infusions were selected because it was shown that fermenting solutions of organic matter are attractive to *Aedes* mosquitoes [9,33]. We also tested alfalfa tablets, which were previously used in ovitraps as an odor lure [10]. We selected tablets with yeast as the main ingredient as an attractant because of its carbon dioxide production and enhancement of bacterial growth in water, and tea capsules as an easily available organic substance with similar characteristics to yeast.

Comparisons between tap water and these attractants were undertaken with trap prototype A (Figure 1A) in a free-flying cage (Howitec Netting BV, Bolsward, The Netherlands, Figure 2B). To measure mosquito attraction we added 200 μl of Aquatain (Aquatain Products Pty Ltd, Kyneton, Victoria, Australia) to 300 ml of the odor-baited water sample and to the control. Aquatain™ (polydimethylsiloxane 78% w/v) is a liquid that creates a monomolecular film on water, which lowers the water surface tension. This silicone oil film has been shown to cause female mosquitoes to drown whilst ovipositing [34], meaning that it can be used to determine the first choice of oviposition location for *Ae. aegypti*.

Table 1 Selected odor lures and method of preparation for experiments

Attractant	Preparation
AtrAedes tablets	1 AtrAedes tablet placed in the water of the trap
Oak leaf infusion	Several oak leaves were placed in a bucket with 8 L of boiling water; then left for 2-4 weeks
Earl grey tea	3 teabags were placed in a bucket with 8 L of boiling water
Organic water	Water collected from a local ditch and stored in a bucket for 2 weeks
Alfalfa (tablets)	4 tablets were placed in a bucket with 8 L water and stored for 4 days
Yeast (tablets)	4 tablets were placed in a bucket with 8 L water and stored for 4 days
Green tea (capsules)	4 capsules were placed in a bucket with 8 L water and stored for 4 days

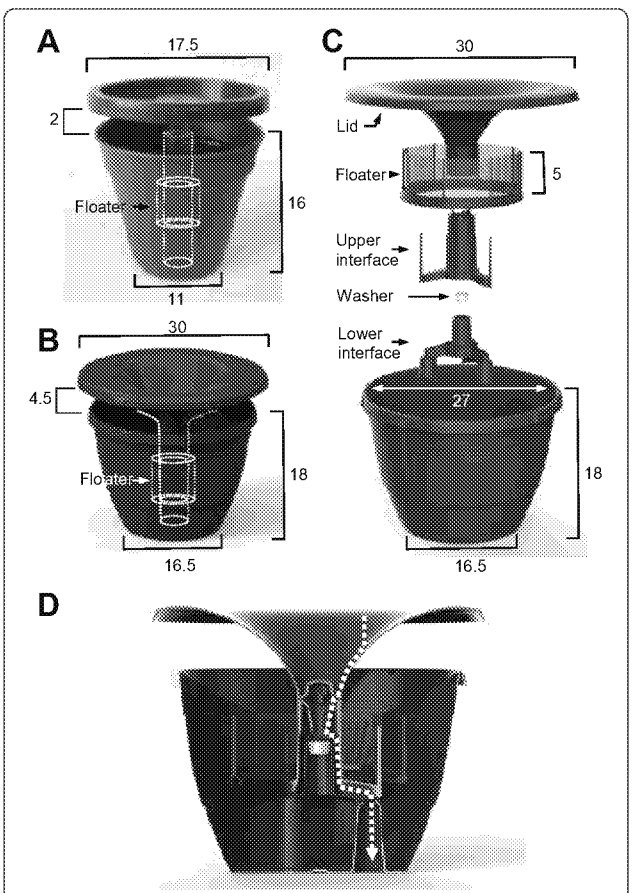

Figure 1 In2Care® Mosquito Trap design. A and **B**: Tested prototypes and position of the central tube and floater (dimensions in cm). **C**: Final design of the In2Care® Mosquito Trap. **D**: Cross section of the trap showing water flow (white arrow) with use of the washer. (Rain) water enters through the lid (blue), into the upper interface (green). The washer prevents the water from filling up the trap so that it leaves the trap trough the lower interface (red) and the drainage openings (yellow).

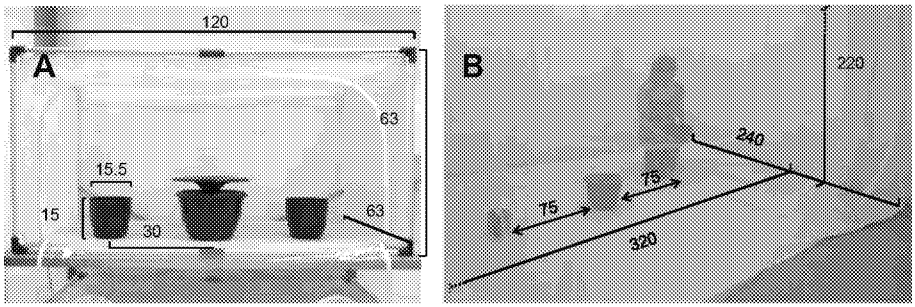

Figure 2 Experimental cages (dimensions in cm) and position of traps and containers. A: Free-flight cage used for the experiments with mosquitocidal agents. **B**: Free-flight cage used for the evaluation of oviposition attractants and floater designs. All cages were kept under similar climate conditions (27(± 1)°C, 65(± 5)% RH).

The trap containing the odor bait was placed on one side of the cage and a trap filled with tap water was placed on the opposite side of the cage. For each comparison fifty gravid *Ae. aegypti* (7-8 days old; four replicates) were released in the cage and positions were switched between the replicates to minimize position effects. After 3 days the number of drowned mosquitoes in each trap was counted to determine their preferred oviposition site. For the comparison between yeast tablets, alfalfa tablets and green tea capsules 3 traps were placed in a row, and the same experimental procedure was followed.

Optimizing floater design

The floater was tested and improved using trap prototype B (Figure 1B). The trap was placed in the center of the cage (Figure 2B) and filled with 2 L water and a yeast tablet. The floaters supported black polyester gauze (Van Heek BV, Losser, The Netherlands) that was dusted with fluorescent powder (BVDA International BV, Haarlem, the Netherlands) and carefully placed on the water surface in the center of the trap. Two alternative breeding sites were positioned in the cage at opposite sides of the trap to provide competitive breeding sites. These consisted of a black pot in which a transparent plastic container with tap water (500 mL) was placed. For Floater-I and Floater-II 3 replicates were conducted, for Floater-III, Floater-IV and Floater-V 4 replicates were conducted. Fifty mosquitoes were allowed to oviposit for 2 days, after which they were recaptured using a mouth aspirator. These were killed in the freezer and the presence of fluorescent powder on the legs and body determined using a UV light microscope. The proportion of mosquitoes with fluorescent dye was used as a proxy for trap visitation and contact with the gauze on the floater.

Control agents

We used *Beauveria bassiana* spores from the GHA strain (Laverlam international corporation, Butte, USA), which were produced through solid-state fermentation. Dried spores were kept at low humidity at 5°C until use. Pyriproxyfen (Chemos GmbH, Regenstauf, Germany) was mixed with fungal spores and inert dust particles to create a dust mixture suitable for application on the gauze. This mixture was used inside the final design of the trap (Figure 1C). The powders were applied to the gauze by shaking it in a container with an excess amount of the powder mixture. We deployed 5 × 55 cm strips of gauze dusted with the mixture, which were subsequently fixed around the pins of the floater (Table 2, Type-V). One trap and 2 alternative breeding sites, each containing water with 20 *Aedes* larvae (stage L4) and Tetramin® fish food were placed in a free-flying cage (MegaView Science, Taiwan, Figure 2A). Two plastic cups with tap water, 20 larvae and fish food, were placed outside the experimental cage as a control treatment to measure adult emergence. For each experiment, 50 free-flying gravid *Aedes* females were allowed to oviposit for 2 days, after which they were recaptured. To measure adulticidal impacts of the fungus, 8 control replicates and 8 replicates with *B. bassiana* were conducted and adult survival was monitored for 18 days. Pyriproxyfen dissemination was tested by measuring larval development (% adult emergence) in the two containers next to the trap and compared to adult emergence from control containers, 4 replicates were conducted for the pyriproxyfen tests.

Statistical analyses

Statistical analyses were done using SPSS 21.0 software. Normality of the data was investigated using the Shapiro-Wilk Test, a Log_{10} transformation was used if data was not normally distributed. Homogeneity of variances was tested with Levene's Test (untransformed data). Comparisons between oviposition attractants were done using independent sample T-tests for normally distributed data and a Mann-Whitney U test for data that were not normally distributed. The comparison between tea capsules, alfalfa tablets and yeast tablets was performed using a

Table 2 Measurement of floaters and position of gauze on the floater, as tested in the floater optimization tests

Floater type	Outer ø* in cm	Inner ø* in cm	Height in cm	Position of gauze
Floater-I (small)	5,5	3,5	6,5	Outer ring of floater covered with gauze
Floater-II (medium)	10,0	3,5	5,0	Outer ring of floater covered with gauze
Floater-III (large)	16,0	3,5	5,0	Outer ring of floater covered with gauze
Floater-IV (ring)	16,0	12,0	5,0	Outer and inner ring of floater covered with gauze
Floater-V (pins)	16,0	14,0	1,5	Gauze on top of floater, stabilized with pins

*Ø = diameter.

one-way ANOVA test followed by a Tukey post-hoc test. Analyses of the floater optimizations and the autodissemination impact were done using a one-way ANOVA test followed by a Tukey post-hoc test. The impact of *B. bassiana* on adult mosquitoes was analyzed with a Kaplan-Meier model followed by analysis with the logrank test. LT_{50} data (median lethal time) was obtained from the survival analysis. Replicates were pooled for both controls and fungus groups. Survival curves of infected groups were compared to control groups.

Results

Device design and optimization

Trap design development started with a simple flowerpot (Figure 1A, prototype A), comprising a black water container, a central tube and a black lid. The black container provides a visually attractive and sheltered breeding site for Dengue mosquitoes and is commonly used for ovitraps [7,15]. Over time, the volume of the container was increased so that it could contain 3 L of water maximum, which allowed for longer trap use and less frequent maintenance (Figure 1B, prototype B). The final design, a stackable pot with 3 water drainage openings in the bottom, is based on a mass-produced, and therefore low-cost, flower pot (Epla Nora-pot, Desch PlantPak, the Netherlands). The inside of the container is made from smooth, polished polyethylene, to discourage egg-laying mosquitoes to rest on this surface, thereby increasing the chance that they will land on the gauze treated with control agents.

We developed a removable lid to protect the control agents against direct sunlight and rainwater, to allow access for maintenance purposes, and to prevent direct contact between children and/or pets and the bioactives on the gauze. In later development stages (prototype B), the diameter of the lid was increased with a slight overhang to provide better protection against heavy rain. Our tests showed that mosquitoes have a preference for an entry opening between the lid and pot of 4-6 cm (data not shown).

The shape of the original flat-top lid (prototype A) was adjusted to prevent the accumulation of stagnant water on top of the lid, which could potentially form a mosquito breeding site. We added a central lid opening that allows replenishment with water and optional collection of rainwater via a central hollow tube. The tube has a valve through which (rain) water flows into the trap container, avoiding a low water level due to evaporation. Simultaneously, when the water level rises above a certain point, excess water will flow out of the trap through this valve via the central tube, which connects to the pot drainage openings in the bottom. The final design (Figure 1C, the 'In2Care® Mosquito Trap') contains a click-on interface that is put on top of the drainage openings, and has 3 protruding extensions that stabilize the floater component.

The floater component was designed to carry vertically placed gauze with mosquitocidal agents and is one of the important novelties of this ovitrap compared to other designs. The In2Care® Mosquito Trap deploys a floater that remains level with the water surface and provides an attractive landing and resting surface for ovipositing mosquitoes. The floater can carry gauze or other materials that are treated or impregnated with mosquitocidal agents and keeps these dry even when water levels fluctuate inside the device.

Evaluation of oviposition attractants

Attractant tests showed an increase in attractiveness of the device when using oak leaf infusions, teabags or organic water (Figure 3). Traps containing these lures were able to catch (i.e. drown due to the presence of Aquatain™) on average more than 80% of all released (free-flying) gravid mosquitoes. Significantly more females drowned in the devices with tea, organic water and oak leaf infusions

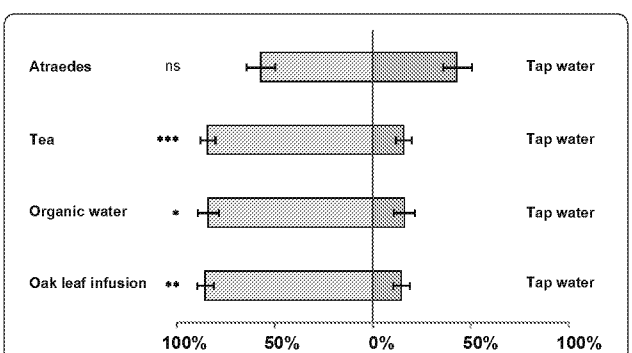

Figure 3 Dual-choice (% ± SE) experiments with different attractants for gravid *Ae. aegypti*. Fifty gravid mosquitoes were released per replicate (4 replicates for each comparison). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

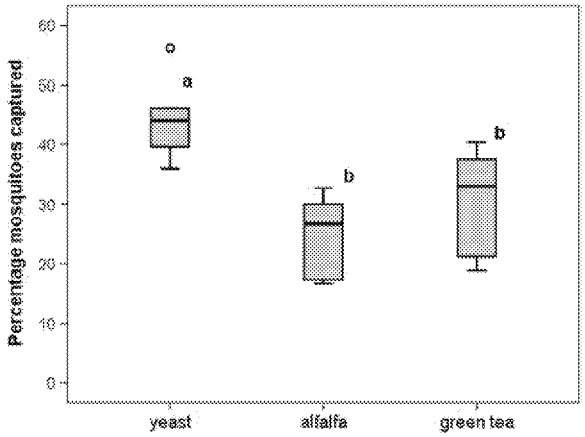

Figure 4 Percentage (± SE) of mosquitoes collected in oviposition traps baited with different odors. Fifty gravid mosquitoes were released per replicate (n = 4). Comparisons were made between the 3 odor-baited traps. Each box denotes the median as a line across the middle and the quartiles (25th and 75th percentiles) at the bottom and top. Treatments without letters in common are significant different at $P < 0.05$. "°" denotes an outlier.

compared to clean tap water ($p < 0.001$, $p = 0.029$ and $p = 0.005$, respectively). This demonstrates that these organic odors are attractive to gravid *Ae. aegypti*. AtrAedes tablets, however, did not show an effect when used in our setup ($p = 0.273$). Because the AtrAedes tablets did not increase trap attractiveness, we selected other commercially available tablets for further tests. We examined the most attractive odor (tea) and looked for commercially available tablets or capsules.

We compared the attractiveness of tea capsules to two other ready-to-use attractants, namely yeast tablets and alfalfa tablets. Yeast was found to be significantly more attractive when deployed in the ovitrap compared to alfalfa (Figure 4, $p = 0.001$) and green tea ($p = 0.018$). On average, 45% of the free-flying mosquitoes selected the yeast-baited trap as their first oviposition site compared to the other two odor lures. No significant difference was found between alfalfa and green tea tablets.

Because yeast tablets were significantly more attractive compared to the tested other odor lures and because the tablets are commercially available, we selected the yeast tablets as the standard oviposition attractant in the trap.

Floater optimization

To maximize mosquito infection and contamination inside the trap, we evaluated and optimized the design of the floater component. We validated the attractiveness of the gauze-carrying floater by measuring mosquito contact using fluorescent dust. We tested different floater types and sizes (Table 2), in experimental cages where 50 gravid mosquitoes were released and retrieved after 2 days to observe the presence of fluorescent powder on the mosquitoes using a UV light microscope. Since only the gauze was dusted with fluorescent powder, the presence of this powder on mosquitoes was used as a measure for contact with the floater gauze and served as a proxy for the attractiveness and efficacy of the floater components. Gauze with fluorescent dust and mosquitoes with fluorescent particles are shown in Figure 5.

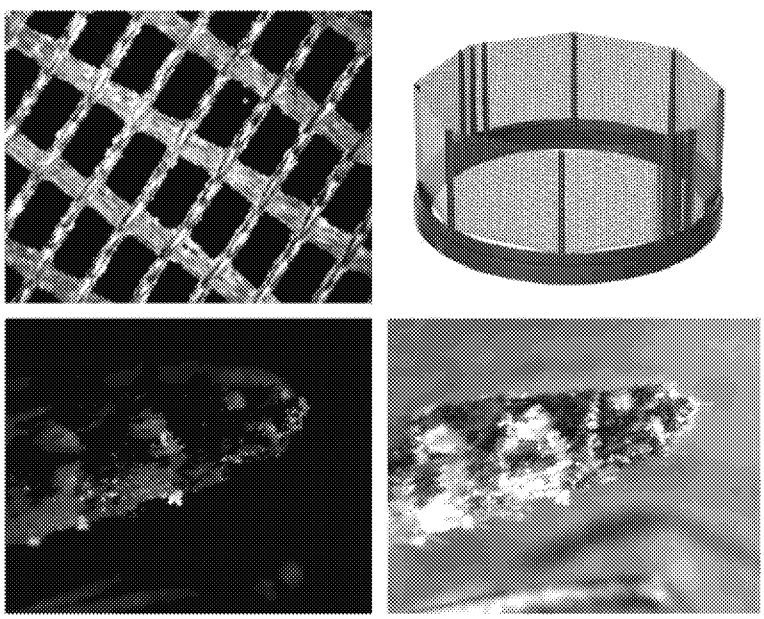

Figure 5 Electrostatic gauze dusted with orange fluorescent powder under UV light (top left), floater-V with gauze (top right), the abdomen of an *Ae. aegypti* exposed to gauze with fluorescent powder (bottom left (UV) and bottom right). Magnification 200x.

Results showed high percentages of mosquitoes with fluorescent powder in particular for the floaters with a large gauze surface (Figure 6). Significant differences in the attractiveness and mosquito contact of the different floaters were observed. The percentage of mosquitoes with fluorescent powder was significantly higher for Floater-IV and V compared to Floater-I ($p = 0.025$ and $p < 0.001$, respectively) and Floater-II ($p = 0.030$ and $p < 0.001$, respectively). Floater-V was also more attractive than Floater-III ($p = 0.003$). Overall, the increase in gauze surface on the floaters increased the percentage of mosquitoes with fluorescent powder (Figure 6). This indicates that the floater provides effective contact and powder transfer to resting/ovipositing mosquitoes and can be used to contaminate these once inside the trap. In all experiments, we observed much higher numbers of mosquito eggs laid inside the prototype device compared to the alternative sites, which indicates that the In2Care®Mosquito Trap is more attractive to gravid *Ae. aegypti* than the open black flower pots.

The final floater design (Floater-V, Table 2) was effective in contaminating, on average, 90% of the retrieved mosquitoes. This design is based on a thin polyethylene ring that floats via five air-chambers in the bottom and has protruding pins onto which the gauze can be fixed (Figure 5). This design allows the use of control agents on both sides of the gauze and enables egg-laying mosquitoes to sit close to the water surface. We therefore selected this floater as the standard floater for the trap.

Mosquitocidal agents

Multiple experiments were conducted to test and improve the impact of mosquitocidal agents in the trap.

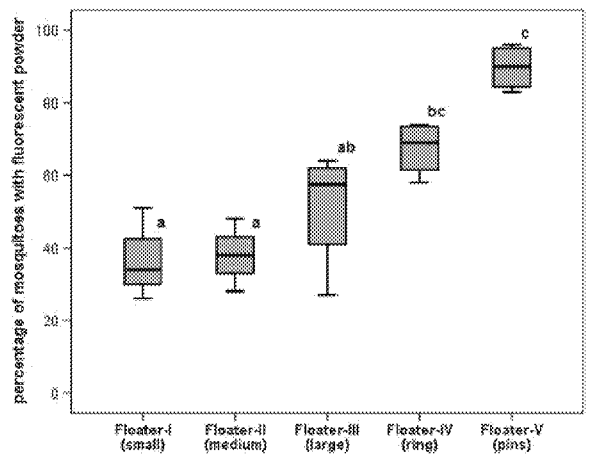

Figure 6 Percentage (± SE) of retrieved mosquitoes with fluorescent powder. Comparisons were made between 5 different floaters in prototype B (see Figure 1). n = 3 for Floater-I and Floater-II, n = 4 for Floater-III, Floater-IV and Floater-V. Each box denotes the median as a line across the middle and the quartiles (25th and 75th percentiles) at the bottom and top. Treatments without letters in common are significant different at $P < 0.05$.

Gauze strips were dusted with a mixture of *Beauveria bassiana* spores and pyriproxyfen particles (as described in more detail in the Methods section) and applied inside the trap using Floater V.

Impact of *Beauveria bassiana* on adult mosquitoes

Mosquitoes retrieved from cages with the trap showed a reduced survival compared to control groups. The Kaplan-Meier LT_{50} estimation of the mosquitoes infected with *Beauveria bassiana* spores was 14.00 days (13.21-14.80, 95% CI, Figure 7). The LT_{50} of control groups was beyond the 18 days measured so could not be calculated with the Kaplan-Meier model.

Survival curves were significantly different for mosquitoes infected with the fungus compared to controls ($p < 0.001$, Kaplan-Meier with logrank test). This impact on adult survival demonstrates that the *B. bassiana* spores applied on the floater gauze are effective in contaminating *Aedes* mosquitoes with high infection doses even with uncontrolled, realistic and potentially short exposure times (Figure 7). The relatively slow killing process of the fungus enables the contaminated females to spread pyriproxyfen to other breeding sites.

Impact of pyriproxyfen on larvae

Results showed that pyriproxyfen was actively dispersed from the trap to the surrounding breeding sites via the contaminated mosquitoes; killing on average >90% of all developing larvae in these sites. This autodissemination impact significantly reduced the emergence of adult mosquitoes in the breeding sites around the device (less than 1 in 10 larvae survived to adulthood) compared to control larvae of which ca. 95% larvae developed into adults (Figure 8, $p < 0.001$, one-way ANOVA test). Furthermore, we found a significant reduction in larval

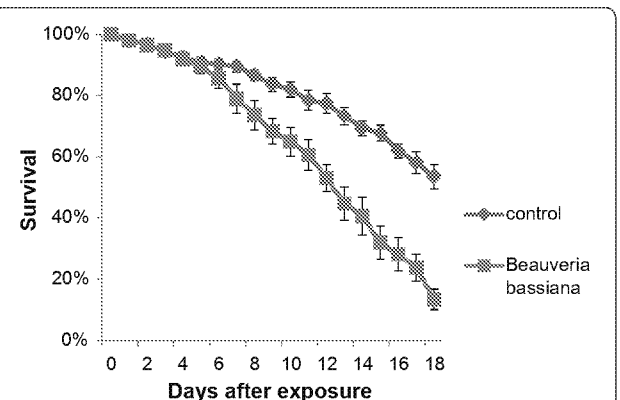

Figure 7 Survival curves (± SE) of mosquitoes exposed to *Beauveria bassiana* (n = 8) deployed in the In2Care mosquito Trap, compared to a control group (n = 8). Adult survival was monitored daily for 18 days after recapture. Survival curves were significantly different at $P < 0.001$.

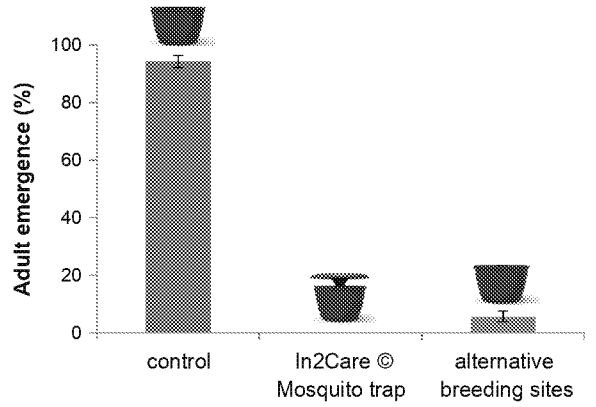

Figure 8 Adult emergence (% ± SE) in dissemination experiments. Emergence was significantly ($P < 0.05$) reduced in the In2Care Mosquito Trap and the alternative breeding sites ($n = 4$) compared to the control larvae ($n = 4$).

survival inside the trap compared to the control larvae ($p < 0.001$, one-way ANOVA test). We consistently observed 100% mortality of the larvae in the trap due to the presence of pyriproxyfen in the water of the trap. It is noteworthy that no additional pyriproxyfen was added to the water in the trap or the alternative breeding sites, which demonstrates that the amount that is transferred into the water solely via visiting ovipositing mosquitoes is sufficient to effectively kill larvae.

Discussion

The In2Care® Mosquito Trap was designed to provide a novel tool to control *Ae. aegypti* mosquitoes. The trap has the advantage of operating without the need for electricity or carbon dioxide and is made from low-cost polyethylene, which makes it a relatively cheap tool for mosquito control. The trap design is suitable for high throughput manufacturing and utilizes commercially available ingredients.

Results showed that this yeast-baited water-filled ovitrap can effectively attract gravid *Ae. aegypti*. Furthermore, the use of pyriproxyfen is expected to further increase mosquito attraction. Because pyriproxyfen is a late-stage killing agent, targeting the pupal stages only, the deposited eggs inside the device will still hatch and develop into larvae which results in the accumulation of larval odors in the trap over time. Studies show that volatiles emitting from larvae are attractive to gravid Dengue vectors [12], and can therefore be expected to augment the attractiveness of the trap.

While most vector control tools focus on either adults or larvae, the In2Care® Mosquito Trap targets both larval and adult life stages of *Ae. aegypti*. Results showed that the powders applied in the trap exert effective mortality impacts on contaminated adult mosquitoes, in the larvae developing inside the trap, as well as the on larval development in surrounding breeding sites. Results also showed that gravid females pick up lethal doses of control agents upon short and transient contact with the floater gauze. The use of fungal spores was selected as an environmentally-friendly alternative to chemical pesticides. The relatively slow mode of action of the fungus *B. bassiana* provides a long-lasting adult control option by targeting only the older females that can transmit disease, thereby drastically reducing the chances for development of resistance [35]. Another major advantage of this fungus is that it causes a reduction in Dengue virus transmission via interference with virus replication inside the mosquito [30]. Other pre-lethal transmission-blocking effects observed in fungus-infected adults include a reduced fecundity and blood feeding propensity, which causes a significant reduction in their vectorial capacity. Moreover, the use of *B. bassiana* in the trap allowed the use of the autodissemination agent pyriproxyfen. It was found that pyriproxyfen was highly effective when deployed inside the trap, because in all experiments 100% of the larvae in the trap died at the time of pupation. Furthermore, we found a significant reduction in larval survival in the breeding sites surrounding the trap compared to the control larvae. This proves that pyriproxyfen was effectively dispersed to other breeding sites by free-flying mosquitoes in the experimental cages, which shows the possibility of controlling *Aedes* mosquitoes using the autodissemination effect, previously described by Devine *et al.* [25] and Caputo *et al.* [22]. Because pyriproxyfen does not have a repellent effect or impact on adult mortality, it allows the full exploitation of the skip-oviposition behavior of *Aedes* mosquitoes. Particularly in areas where breeding sites are abundant and transient during the wet season, the use of pyriproxyfen provides an exciting opportunity for precision-targeted larval control using the female mosquito itself.

Potentially, other insecticidal agents could also be used in the device, such as fast-killing chemicals like the carbamate bendiocarb. Investigations on the efficacy of the trap using these and other insecticidal agents are currently ongoing. Whereas most vector control chemicals will have the advantage of a fast killing effect (and thereby the visual confirmation of dead mosquitoes inside the trap), this effect will not be useful when using autodissemination agents, which require the contaminated mosquito to survive for at least a few days to successfully spread pyriproxyfen to surrounding breeding sites.

Although the trap was originally designed to lure *Ae. aegypti* mosquitoes, we have seen *Culex* species resting inside the trap during field trails. These observations show good potential for further investigations on the attractiveness of the trap for other mosquito species (for example other culicines and maybe even anophelines). It

has been shown that multiple disease transmitting mosquito species can be targeted using ovitraps, such as *Ae. albopictus* [7,15,16], *Cx. quinquefasciatus* [16,17,36] and *Cx. pipiens* [37]. In future studies, we will evaluate additional lures to attract other species, which could improve pyriproxyfen dissemination coverage and broader uptake of this control tool. Large-scale field tests with epidemiological outputs are the next logical step in this research. Additional field validations will be needed to further examine and augment the attractiveness of the trap in a more natural situation, and to gain more knowledge on the optimal placement and deployment of the traps in a variety of settings.

Conclusions

The trap described in this work can have an advantage over existing control tools in integrated vector control strategies aimed at significantly reducing *Ae. aegypti* populations, because of the unique multi-dimensional effect of trap. Like other ovitraps, the trap does not provide personal protection, which means that additional measures to prevent disease transmission will be needed. However, because the trap also has an effect on breeding sites in the vicinity of the trap it could be a useful tool in integrated vector management campaigns to provide protection around the house and in public places. Large-scale deployment and proper placement and servicing of the trap will be needed to be able to reduce Dengue vector densities and disease transmission. Trap servicing will be needed every 6-8 weeks, to refill the trap with water, add an odor tablet and to replace the gauze with control agents. Even with this frequent need for servicing, the trap's low cost price makes it a promising addition to current Dengue vector control tools.

Competing interests

All authors are remunerated and/or employed by In2Care BV and have commercial stakes with regard to the device described in this article.

Authors' contributions

JS and RA contributed to the experimental designs, conducted laboratory experiments, performed the data analysis and JS drafted the manuscript. RAS contributed to the experimental designs and AJO contributed with device developments and improvements. BGJK and MF designed the experiments and revised the manuscript. All authors read and approved the final version of the manuscript.

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Electrostatic coating enhances bioavailability of insecticides and breaks pyrethroid resistance in mosquitoes

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Insecticide resistance poses a significant and increasing threat to the control of malaria and other mosquito-borne diseases. We present a novel method of insecticide application based on netting treated with an electrostatic coating that binds insecticidal particles through polarity. Electrostatic netting can hold small amounts of insecticides effectively and results in enhanced bioavailability upon contact by the insect. Six pyrethroid-resistant *Anopheles* mosquito strains from across Africa were exposed to similar concentrations of deltamethrin on electrostatic netting or a standard long-lasting deltamethrin-coated bednet (PermaNet 2.0). Standard WHO exposure bioassays showed that electrostatic netting induced significantly higher mortality rates than the PermaNet, thereby effectively breaking mosquito resistance. Electrostatic netting also induced high mortality in resistant mosquito strains when a 15-fold lower dose of deltamethrin was applied and when the exposure time was reduced to only 5 s. Because different types of particles adhere to electrostatic netting, it is also possible to apply nonpyrethroid insecticides. Three insecticide classes were effective against strains of *Aedes* and *Culex* mosquitoes, demonstrating that electrostatic netting can be used to deploy a wide range of active insecticides against all major groups of disease-transmitting mosquitoes. Promising applications include the use of electrostatic coating on walls or eave curtains and in trapping/contamination devices. We conclude that application of electrostatically adhered particles boosts the efficacy of WHO-recommended insecticides even against resistant mosquitoes. This innovative technique has potential to support the use of unconventional insecticide classes or combinations thereof, potentially offering a significant step forward in managing insecticide resistance in vector-control operations.

electrostatic coating | insecticide | resistance management | mosquito | malaria

Mosquito-borne infectious diseases continue to pose a huge public health burden worldwide. Malaria, lymphatic filariasis, dengue, Chikungunya, and West Nile virus cause significant medical and economic impacts that disproportionately affect developing countries (1–3). Because there are no commercially available vaccines against these diseases, vector control remains crucial to reduce disease transmission. Contemporary vector control focuses on the use of four classes of public health insecticides recommended by the WHO (4). However, intensive and widespread use of these insecticides induced intense selection pressure that has resulted in the development and subsequent intensification of various genetically modulated resistance mechanisms in mosquitoes (5–7). Today, insecticide resistance is regarded as the most serious threat to the control of mosquito-borne diseases. Over the last decade resistance has been reported in all three major mosquito genera and against all four classes of recommended insecticides in most disease-endemic regions where substantial progress in control was reported previously (4, 8).

Larval exposure to low residual doses of insecticides from agricultural pest control has been a major driver of resistance development in mosquito populations (9, 10). Mosquitoes can become resistant to insecticides by (over)expressing detoxifying enzymes or via genetic mutations at the location where the insecticide is active (11). Such traits might result in fitness costs for the insect because their expression can deplete energy resources, reducing the insect's ability to compete with nonresistant counterparts (12–14). To manage resistance adequately, the WHO recommends the use of rotations, insecticide mixtures, or novel insecticide classes that have completely distinct modes of action (4), and several promising developments are aimed at facilitating these strategies (15–20). However, the selection of new active ingredients is severely restricted by the need for products that are safe for humans who come into frequent contact with nets and sprayed surfaces. Another means to improve insecticidal impact is to increase the effective target dose.

Several factors influence what dose of insecticide is effectively transferred to the target insect, including the type of formulation and substrate as well as the size and adherence properties of

Significance

Conventional mosquito-control methods are becoming less effective in controlling mosquito-borne diseases because of widespread resistance against safe and recommended public health insecticides. Therefore innovative and effective alternatives are urgently needed. We present a novel method for exposing mosquitoes to insecticides that uses electrostatic forces to bind insecticide particles. Results show that this method increases exposure of mosquitoes to such an extent that even those that have developed high levels of resistance can be killed effectively. The ability to boost the efficacy of WHO-recommended insecticides provides a significant benefit to the field of vector-borne disease control and will further the development of novel resistance-breaking control tools.

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Conflict of interest statement: R.A., J.S., R.A.S., A.J.O., B.G.J.K., and M.F. are remunerated by or receive compensation for services delivered to In2Care BV and hold shares in In2Care BV. In2Care BV has one or more patents or patent applications related to the subject of this paper.

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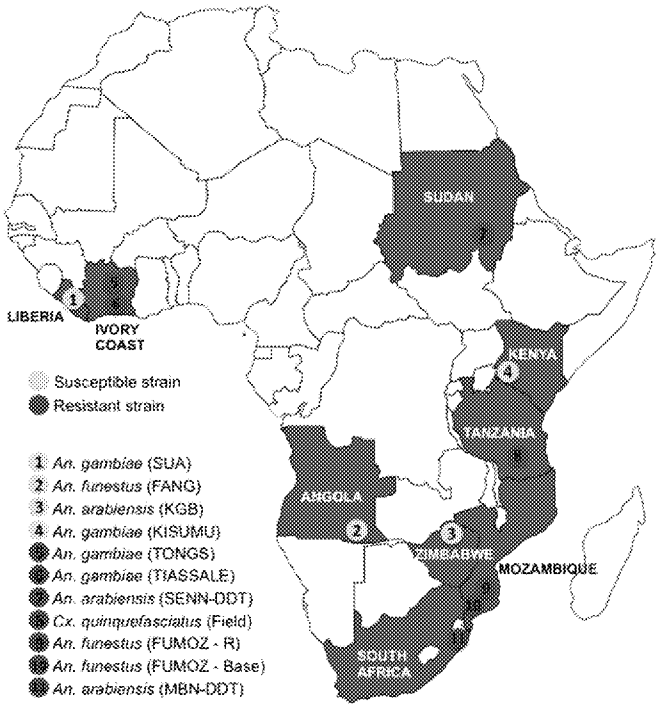

Fig. 1. Origins of the tested pyrethroid-resistant and susceptible *Anopheles* strains and the field-collected *Culex* strain.

insecticidal particles on these substrates (21). Small flying insects such as mosquitoes are particularly difficult to target with lethal doses of insecticides. Current vector-control products use oil- or water-based formulations as carriers to achieve adherence and retention of the chemicals on vertical substrates such as walls or netting. For instance, long-lasting insecticidal nets (LLINs) and indoor residual sprays (IRS) used in malaria control deploy formulations of pyrethroids via coating, impregnation, or spraying. Absorption of the carrier and binding forces associated with oily formulations can limit the bioavailability of the active ingredient. Studies show that insecticide absorption in porous mud walls significantly decreases the long-lasting efficacy of IRS applications (22). For LLINs, impregnation methods such as insecticide incorporation within the netting fibers or slow-release coatings are being used to prolong persistence and withstand several washings, but these techniques limit the amount of insecticide that is available to the target insect upon contact. Prolonged use of long-lasting material under normal household conditions might result in mosquitoes being exposed to a progressively smaller dosage of insecticides as the chemicals dissipate (23); a consequence of such exposure might be selection for resistant vector populations.

Here we present an application method with improved insecticide bioavailability that can be used to deploy both pyrethroid and nonpyrethroid insecticides to target resistant vectors effectively with a lethal dose. This method consists of a coating that can be applied on different substrates and has an electrostatic charge that binds particles via polarity. Products that incorporate electrostatic binding forces are already being used in insect pest control, for example as aerial electrostatic-charged insecticide sprays for the control of sweet potato whitefly (*Bemisia tabaci*) (24) or as electrostatic mating-disruption powder against codling moth (*Cydia pomonella*) (25). However, this is the first time, to our knowledge, that electrostatic forces have been used in a coating that can be deployed on a variety of surfaces and that can bind different types of particles. The charged coating was developed originally for use in house screens to trap airborne pollen and is commercially available for that purpose (https://www.buypollentec.com/test-data/ accessed July 29, 2015). It is applied on netting fibers via a special process that allows fixation and the formation of a long-lasting static charge. The electrostatic charge enables the adherence of insecticide particles without the need of a carrier formulation.

In this study, the bioavailability and impact of insecticide particles bound via polarity were measured and compared with the impact of a standard long-lasting deltamethrin-coated bednet. Multiple strains of pyrethroid-resistant *Anopheles* malaria vectors originating from nine different countries in Africa (Fig. 1) were exposed to electrostatic netting saturated with deltamethrin amounts similar to the insecticide dose used in standard LLINs [PermaNet 2.0; target dose, 55 mg active ingredient (AI)/m^2]. The impact of the two methods was compared by measuring initial knockdown (1 h) and final mortality (24 h post exposure). Lower insecticide doses and shorter exposure times were included to demonstrate the potential vector-control impact of electrostatic netting. Three other (nonpyrethroid) public health insecticide classes were deployed, and the mortality impact against *Aedes aegypti* and *Culex quinquefasciatus* mosquitoes was tested to demonstrate the broader vector-control and resistance-management options of this innovative application technique.

Results

Particle Transfer. To test the bioavailability of insecticide particles, particle transfer to mosquitoes was visualized by applying fluorescent dust on the electrostatic netting. The quantity of transferred fluorescent particles served as a visual proxy for contamination efficacy (Fig. 2*A*). In a standard 3-min WHO cone exposure assay (26), mosquitoes obtained fluorescent particles across the entire body including tarsi, antennae, proboscis, thorax, and lower abdomen (Fig. 2*C*), demonstrating that an extensive dose was transferred from the electrostatic netting to the mosquito. Shorter contact assays, using exposures of only 5 s, also resulted in effective particle transfer from the netting to mosquitoes, with visible amounts of fluorescent particles adhering to the tarsi and body (Fig. 2*B*). Both exposure times were included in the insecticide impact evaluations to quantify insecticidal impacts after standard and short contact duration.

Insecticide Bioavailability. Insecticide exposures were conducted on multiple strains of pyrethroid-resistant mosquitoes, including the major malaria vectors *Anopheles arabiensis* (27–29), *Anopheles funestus* (30, 31), and *Anopheles gambiae s.s.* (32, 33) with well-defined mechanisms and levels of resistance, using standard WHO resistance assays with 0.05% deltamethrin papers (Fig. S1). A detailed description of the mosquito strains used, including their origin and resistance mechanisms, is provided in the *Supporting Information*. Insecticide-susceptible mosquito strains were included to confirm sample quality.

The insecticidal impact of deltamethrin coated on a standard polyester LLIN was compared with deltamethrin applied on electrostatic netting, keeping the amounts of AI as similar as

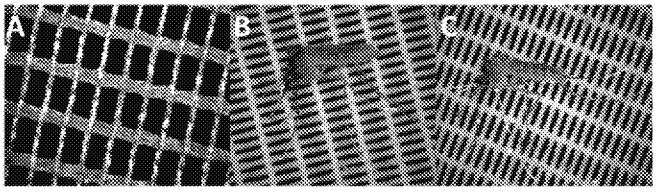

Fig. 2. (*A*) Photograph of electrostatic netting saturated with fluorescent dust particles lighting up orange under UV light at 50× magnification. (*B*) A *Culex* mosquito contaminated with fluorescent particles after a 5-s contact with the netting. (*C*) *Culex* female with fluorescent particles after 3-min contact with netting.

possible. Standard 3-min WHO cone exposure assays were used to compare PermaNet 2.0 netting with deltamethrin (target dose, 55 mg AI/m^2) and electrostatic netting saturated with deltamethrin (target dose, 37 mg AI/m^2). Positive control tests with susceptible anopheline strains showed that both PermaNet and electrostatic netting induced 100% mortality 24 h after exposure, confirming the insecticidal quality of the tested samples (Fig. S2).

Results showed that for all six tested resistant *Anopheles* strains the adulticidal impact of deltamethrin applied on electrostatic netting was significantly higher than the impact induced by LLIN netting ($P < 0.001$ for all groups) (Fig. 3). PermaNet 2.0 killed only 9.6% [95% confidence interval (CI) = 1.2–18.0] of the highly resistant *An. gambiae* Tiassale strain, whereas electrostatic netting with a 33% lower application dose of deltamethrin induced 100% mortality. The most resistant *Anopheles* line tested, *An. funestus* FUMOZ-R, showed 10.6% mortality (CI = 1.8–19.4) after PermaNet exposure, whereas the electrostatic netting was able to knock down 96% (CI = 89.8–100) (Table S1) and kill 63% (CI = 49.1–76.9) of the exposed females (Fig. 3). The significantly higher mortality rates induced by electrostatic netting indicate that higher amounts of insecticide were transferred upon contact. The enhanced bioavailability of electrostatically bound particles thus enables effective killing of resistant mosquitoes with WHO-approved doses of public health insecticides.

Short Contact and Low Insecticide Doses. To test further the potential vector-control capacity of electrostatic netting, the exposure period was reduced, and the dose of test insecticides was lowered. A 5-s exposure time was included to mimic situations in which mosquitoes make only short and transient contact with a treated surface, for instance when netting is applied in eave screens. The impact of exposure on the highly resistant *An. gambiae* Tiassale strain and its susceptible counterpart (Kisumu strain) were tested, as were two strains of *Cx. quinquefasciatus* and one strain of *Ae. aegypti*. All strains except the susceptible Kisumu strain were resistant to LLIN exposure, which killed less than 40% after 24 h (Table S2). A 5-s contact resulted in overall lower knockdown and mortality impacts than the 3-min exposures but confirmed the enhanced bioavailability of deltamethrin on electrostatic netting. With a 5-s exposure, electrostatic netting induced significantly higher mortality rates than LLIN netting: 60–100% mortality ($P < 0.05$ for all groups) (Fig. 4). This increased impact was observed not only in the resistant laboratory strains but also in field-collected *Culex* specimens from the Kilombero valley in Tanzania. That mosquito contact as short as 5 s is sufficient to induce high mortality indicates that the electrostatic coating may be a useful application technique for vector-control tools for which insect–surface contact is short and transient, such as eave screens or lure-and-kill devices.

Furthermore, experiments were conducted to test whether lower-than-standard insecticide doses can be used to target mosquitoes effectively when using electrostatic netting. A 15-fold lower target dose of deltamethrin (3.7 mg AI/m^2) still was able to kill significantly more resistant mosquitoes in standard cone assays than LLIN netting coated with deltamethrin at 55 mg AI/m^2 ($P < 0.001$; Fig. 5). This difference was less pronounced in the *Ae. aegypti* strain that showed minimal resistance to the LLIN. These results show that electrostatic netting can kill highly pyrethroid-resistant mosquitoes with deltamethrin concentrations 93% lower than those used on an LLIN and confirm that applying insecticide particles on an electrostatic coating significantly improves bioavailability and hence mosquito mortality.

Resistance-Breaking Vector-Control Options. A range of nonpyrethroid public health insecticides was tested on electrostatic netting, including bendiocarb (a carbamate) and azamethiphos (an organophosphate). Chlorfenapyr (a pyrrole), which is a relatively new class of insecticide for malaria vector control, was evaluated also (34). With standard 3-min cone assays, all tested insecticides induced 100% mortality in *Cx. quinquefasciatus* and *Ae. aegypti* mosquitoes 24 h after exposure (Table 1). When contact time was reduced to 5 s, the fast-acting chemicals bendiocarb and azamethiphos still induced 100% mortality, whereas the slower-acting chlorfenapyr induced 97% (CI = 89.9–100) and 31% (CI = 15.5–45.7) mortality in *Cx. quinquefasciatus* and *Ae. aegypti*, respectively (Table 1). For both strains, 5-s exposures to chlorfenapyr achieved 100% killing after 48 h. These findings show that the electrostatic coating can be used to apply multiple insecticide classes onto polyester netting effectively and can achieve high mortality rates in mosquitoes from all important vector genera.

Discussion

For the first time, to our knowledge, these data have demonstrated a new technique to apply and retain insecticides on vertical treated surfaces without a carrier formulation. Results show that the uptake of insecticidal particles from electrostatic netting is much more efficient than the uptake from an LLIN at almost similar or lower target doses of active ingredient per unit surface

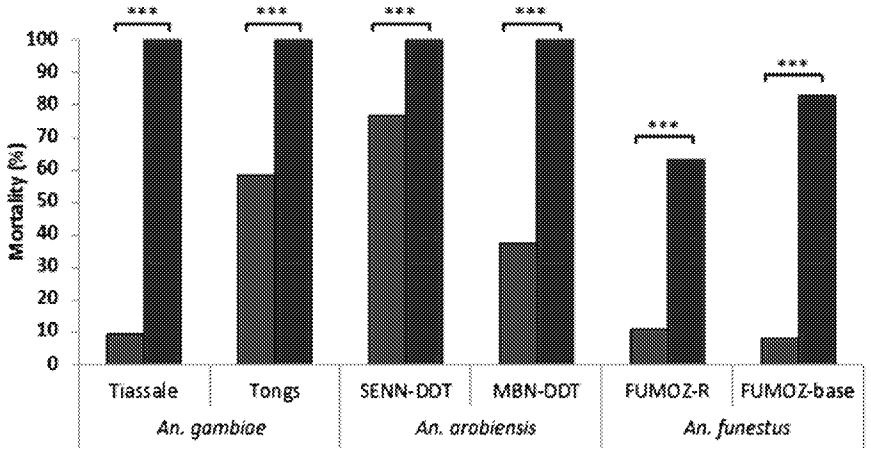

Fig. 3. Corrected mortality percentage (n = 50 mosquitoes per treatment) 24 h after pyrethroid-resistant anopheline mosquito strains were exposed to PermaNet netting (55 mg deltamethrin/m^2; blue bars) or electrostatic netting (37 mg deltamethrin/m^2; red bars) for 3 min. For each treatment, the mortality of mosquitoes exposed to insecticide was corrected for the mortality of counterparts exposed to control netting using Abbott's formula. Asterisks indicate significant differences determined by χ^2 test; ***$P < 0.001$.

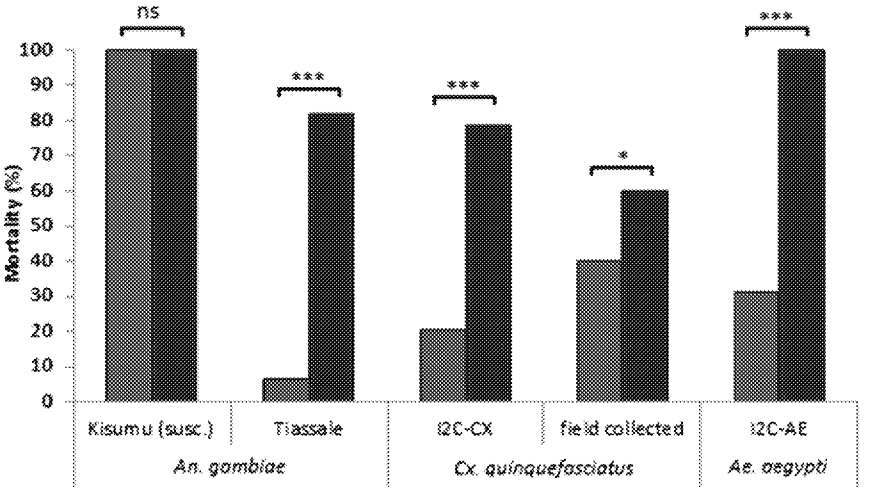

Fig. 4. Corrected mortality percentage ($n = 50$ mosquitoes per treatment) 24 h after pyrethroid-resistant mosquito strains were exposed to PermaNet netting (55 mg deltamethrin/m^2; blue bars), or electrostatic netting (37 mg deltamethrin/m^2; red bars) for 5 s. For each treatment, the mortality of mosquitoes exposed to insecticide was corrected for the mortality of counterparts exposed to control netting using Abbott's formula. Asterisks indicate significant differences determined by χ^2 test; *$P < 0.05$; ***$P < 0.001$; ns, not significant.

area. Fluorescent dust tests provided visual support of high powder-transfer efficacy even upon short and transient contact. High insecticidal efficacy of electrostatic netting against six *Anopheles* mosquito strains with different mechanisms of pyrethroid resistance from across Africa was demonstrated. Even with a mere 5-s contact and at a 15-fold lower dose, the impact of deltamethrin on electrostatic netting was significantly higher than the impact of deltamethrin on an LLIN, confirming the increased bioavailability of the active ingredient. Because the active compound, deltamethrin, was kept unaltered in these comparisons and the observed increases in mortality were similar in all strains regardless of their resistance status, it is unlikely that the mosquitocidal impacts were augmented by differences in modes of action, toxicity, or resistance mechanisms. It is likely that the higher mortality observed results solely from the significant increase in the effective contamination dose, which exceeds the dose that can be tolerated by resistant strains. Similar results indicating that higher dosages of permethrin kill resistant genotypes more efficiently than lower dosages have been reported previously (35). This innovative application method presents an opportunity to improve greatly the control of malaria mosquitoes, in particular those that have become resistant to the insecticides recommended by the WHO.

Netting is a useful application surface for targeting mosquitoes because it can be deployed in house-screening tools and bednets. Currently, insecticide applications for polyester, polyethylene, or polypropylene netting fibers are limited to pyrethroids, the only class of insecticides that can withstand the high temperatures involved in the impregnation process and considered safe enough when contacted daily by humans to be used in bednets. By using the electrostatic coating, we were able to apply multiple public health insecticide classes onto polyester fibers successfully. The

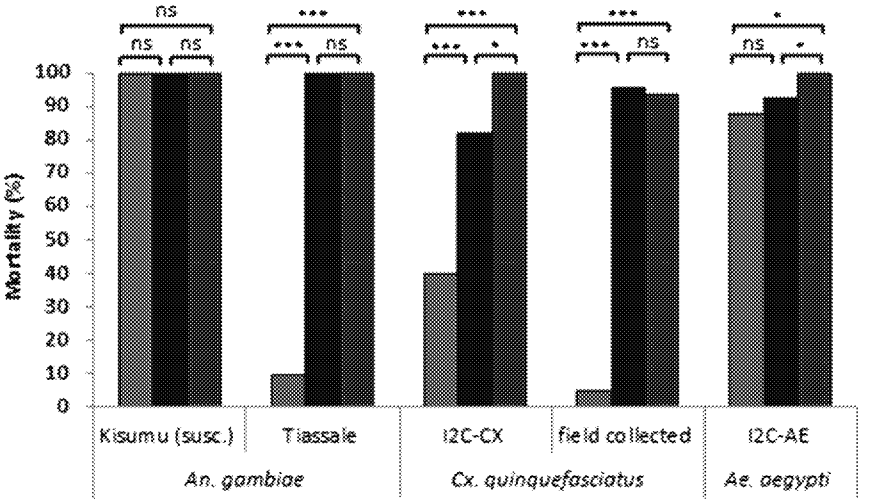

Fig. 5. Corrected mortality percentage ($n = 50$ mosquitoes per treatment) 24 h after pyrethroid-resistant mosquito strains were exposed for 3 min to PermaNet netting coated with deltamethrin (55 mg AI/m^2; blue bars), a 15-fold lower dose of deltamethrin on electrostatic netting (3.7 mg AI/m^2; black bars), or a similar dose of deltamethrin on electrostatic netting (37 mg AI/m^2; red bars). For each treatment, the mortality of mosquitoes exposed to insecticide was corrected for the mortality of counterparts exposed to control netting using Abbott's formula. Asterisks indicate significant differences determined by χ^2 test; *$P < 0.05$; ***$P < 0.001$; ns, not significant.

Table 1. Corrected knockdown and mortality in exposed *C. quinquefasciatus* or *A. aegypti* mosquitoes after contact with insecticide-loaded electrostatic-coated gauze

			10% azamethiphos			20% chlorfenapyr			1.25% bendiocarb		
Mosquito genus	Strain	Exposure time	*N*	Knockdown, % (95% CI)	Mortality, % (95% CI)	*N*	Knockdown, % (95% CI)	Mortality, % (95% CI)	*N*	Knockdown, % (95% CI)	Mortality, % (95% CI)
Culex	I2C-CX	5 s	32	87.5 (76.1–98.9)	100	29	3.4 (0.1–10.1)	96.6 (89.9–100)	48	100	100
	(laboratory)	3 min	48	100	100	44	39.0 (72.7–93.9	100	47	100	100
Aedes	I2C-AE	5 s	48	83.3 (72.7–93.9)	100	36	0.0	30.6 (15.5–45.7)	50	92.0 (84.6–99.4)	100
	(laboratory)	3 min	46	100	100	53	0.0	100	44	100	100

Corrected knockdown and mortality in percentage of *C. quinquefasciatus* or *A. aegypti* mosquitoes after 5-s or 3-min contact with electrostatic coated gauze loaded with public health insecticides in powder form. *N* indicates the total number of mosquitoes exposed per strain (in groups of five to eight mosquitoes). Knockdown and mortality rates are shown with 95% CIs calculated for each pooled sample proportion.

ability to deploy multiple insecticides effectively against all important mosquito vector genera opens a myriad of resistance-breaking opportunities to improve the impact of vector control in areas where insecticide resistance is a problem. The electrostatic coating is not limited to insecticidal applications. Previous studies show that it can be used effectively to apply novel and biological control agents such as entomopathogens and autodisseminants. Experiments with *Beauveria bassiana* spores and pyriproxyfen applied on electrostatic netting inside a novel *Aedes* ovitrap showed that effective doses of these agents were transferred to gravid aedine mosquitoes, inducing high fungus infection rates and successful larvicide dissemination (36). Electrostatic netting thus can provide a means to apply and deploy novel insecticides currently under development (16), which can further assist in the management of insecticide resistance. Further experiments will focus on using combination products, such as multiple classes of insecticides and combinations with potentially synergistic biological agents (37, 38), and on determining particle characteristics to investigate binding and retention on the electrostatic netting. In-depth knowledge of the binding strength of various types of insecticidal particles to the coating might result in further optimization of electrostatic netting for insect control.

This application technique has potential for use in a large variety of vector-control tools. The electrostatic coating can be applied effectively onto various surfaces, including walls, via spray or paint. Quality control tests with the electrostatic coating on antipollen screens have shown that the electrostatic netting fibers can be washed up to 40 times and still retain the electrostatic charge. Thus coated surfaces can remain active for long time periods and can be reloaded with insecticide at appropriate time intervals. This feature may be of particular use for IRS-like wall applications and tools that use removable inserts. However, further research and field tests will be needed to demonstrate the impact and utility of the coating on different (re)treated substrates and in novel vector-control products.

The electrostatic coating is not considered suitable for bednet treatment, because WHO approves only pyrethroid impregnations for such products (39), and reduced efficacy with direct contact and repeated handling of the netting is anticipated. Electrostatic netting can be useful for house-screening products and point-source applications such as mosquito traps (36). The use of electrostatic netting placed at the eave level of rural houses in Tanzania is currently being investigated. In these houses, the eaves are sealed, and 6-in eave tubes are inserted into the wall. Mosquitoes attracted to the human odors that pass through the tubes are blocked from house entry by electrostatic netting that covers the eave tube. Ongoing field studies indicate that bendiocarb- and deltamethrin-saturated electrostatic netting is highly effective when placed in eave tubes. Large-scale field tests in an area with high frequency of insecticide resistance and particularly multiple insecticide resistance, such as West Africa, would be a next logical step in this research.

When electrostatic netting is deployed, even 15-fold lower doses of approved public health insecticides can kill resistant mosquito strains effectively. The electrostatic mode of application thus may provide a means to lower the total amount of AI needed for effective vector control. More studies will be needed to measure the extent to which doses can be lowered for different insecticides while still achieving resistance-breaking impacts. The potential ability to reduce insecticide application doses in mosquito-control tools could help reduce negative impacts on human health and the environment and might provide more cost-effective vector-control options; such options currently are needed in disease-endemic regions where resources are limited.

In conclusion, the application of electrostatically adhered particles can boost the efficacy and provide resistance-breaking applications of currently recommended public health insecticides. Electrostatic netting offers a wide range of application options for vector control, potentially using insecticide combinations or mosaicking/rotations of multiple bioactives and/or novel classes of insecticides. Insecticide resistance is a growing problem in many countries; a new application technique that can boost insecticidal impact, reduce application doses, and expand options for using other bioactives will provide a significant step forward in vector control and resistance management.

Materials and Methods

Mosquitoes. Mosquito strain specifications, origins, and resistance profiles are listed in Tables S3 and S4. *Ae. aegypti* and *Cx. quinquefasciatus* strains were reared and maintained under laboratory conditions at In2Care BV. *An. gambiae* strains were reared under laboratory conditions at the Liverpool School of Tropical Medicine or at the Vector Control Reference Laboratory in Johannesburg, South Africa. *Cx. quinquefasciatus* specimens were collected as larvae from septic tanks in the field in Ifakara, Tanzania (8.05592 S, 36.41001 E) in March 2014 and were kept under ambient conditions. To exclude the impact of intrinsic factors related to insecticide toxicity, all mosquito cohorts used comprised 2- to 6-d-old unfed females, according to the WHO protocol (40).

Anopheline Resistance Levels. The deltamethrin-resistance status of the anopheline mosquito strains tested in the Vector Control Reference Laboratory in Johannesburg, South Africa was confirmed. Standard WHO resistance assays were performed, comprising a 1-h exposure of two replicate groups of 25 unfed females (2- to 4-d-old) per strain, using test tubes lined with 0.05% deltamethrin papers obtained by the WHO vector-control reference unit in Malaysia. The susceptible strains (SUA, TONGs, KGB, and FANG) were included to confirm the insecticidal activity of the WHO test papers (Fig. S1). The susceptible strains all showed 100% knockdown after 1 h and 100% mortality 24 h after exposure. Results of the knockdown and mortality rates of the resistant lines are shown in Tables S1 and S2.

Insecticides. For baseline pyrethroid impacts, bioavailability tests used a standard WHO-recommended bednet, PermaNet 2.0 (Vestergaard-Frandsen) (41), a long-lasting insecticide-treated polyester net that contains coated deltamethrin (target dose, 55 mg AI/m^2) obtained through the courtesy of Helen Pates, Vestergaard-Frandsen, Lausanne, Switzerland. To evaluate electrostatic netting samples, we used a deltamethrin powder (Spritex antiwasp powder

containing 0.25% AI) produced by Denka International BV. Electrostatic netting was manufactured by Van Heek Textiles BV. Netting samples of 15 × 15 cm were fully saturated with deltamethrin powder by manually shaking an excess of powder on the gauze in a closed container, resulting in a target dose of 37 mg AI/m^2 gauze. A dilution of deltamethrin dust was prepared by mixing 10% Spritex antiwasp powder and 90% inert dust (synthetic amorphous silica; van Eck BV). This dilution was applied onto netting by the same application method, resulting in a target dose of 3.7 mg AI/m^2. Untreated polyester bednet material (Vestergaard Frandsen) and untreated electrostatic polyester netting (Van Heek Textiles BV) were included as control treatments.

Bioassays with other public health insecticide classes deployed 1.25% bendiocarb dust (Ficam D; Bayer), 10% azamethiphos wettable powder (Twenty One WP), and 20% chlorfenapyr powder (technical-grade chlorfenapyr obtained from CTF2000) mixed in inert dust. These tests included untreated electrostatic polyester netting as control treatments.

Exposure Assays. Three-minute exposures were conducted using cone assays according to the WHO protocol (40). Short-contact assays were conducted using a similar set-up made from 1.5-L plastic bottles with the lower half removed and the open end covered with treated netting (42). In all exposures the mosquito numbers and ages were according to the WHO protocol and manual aspirators were used to remove the mosquitoes from the cones after 3-min gauze contact or from the bottles after 5-s gauze contact. After exposure, mosquito cohorts were pooled per treatment, and 1-h knockdown and 24-h mortality were recorded.

Statistical Analysis. If the control mortality ranged between 5% and 20%, mortality data were corrected using Abbott's formula to adjust mortality: $(\%) = (X - Y)/(100 - Y) \times 100$, where X is the percentage mortality in the treated sample and Y is the percentage mortality in the untreated control sample. Data were analyzed using SPSS 21.0 software. For each experiment, treatments were compared using χ^2 tests to test for significance. 95% CIs were estimated for the pooled sample proportions, using the one-sample test of proportions formula: $CI = (\hat{p} \pm z^*\sqrt{(\hat{p}(1-\hat{p})/n)})^*100$, where $\hat{p}$ is the sample proportion, z is the critical value for a 95% CI, and n is the sample size.

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Supporting Information

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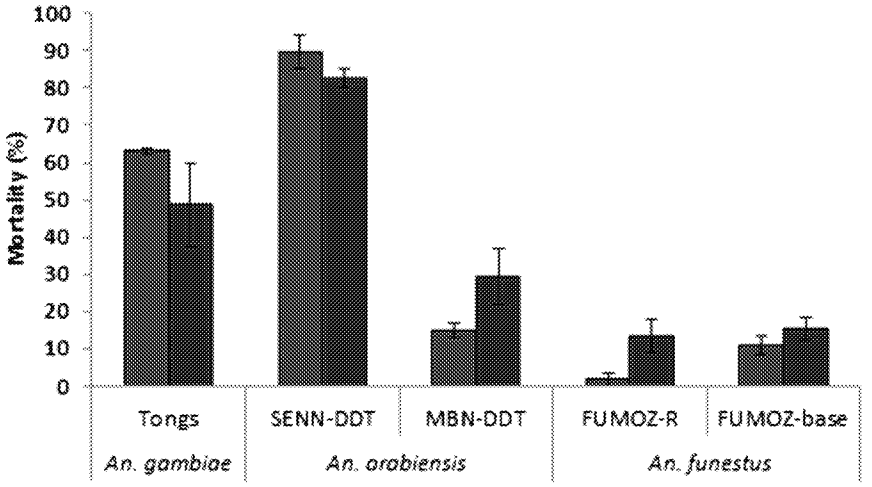

Fig. S1. Average (± SE) mortality and knockdown rates of two groups of 25 female pyrethroid-resistant anopheline strains from the Vector Control Research Unit (VCRU) measured 1 h (blue bars) or 24 h (red bars) after 1-h exposure to 0.05% deltamethrin papers in WHO test tubes.

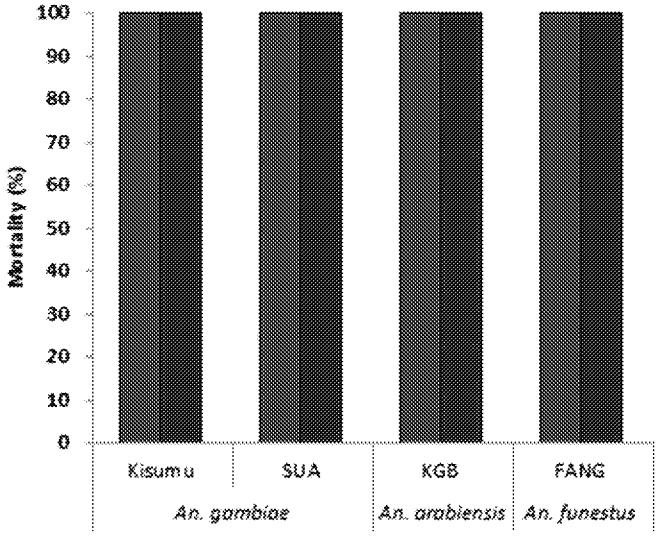

Fig. S2. Mortality rates of susceptible anopheline strains measured 24 h after 3-min exposure to PermaNet 2.0 netting containing 55 mg/m^2 deltamethrin (blue) or electrostatic netting containing 37 mg/m^2 deltamethrin (red).

Table S1. Corrected knockdown and mortality expressed as the percentage of the total number of exposed mosquitoes after 3-min exposure to standard deltamethrin-coated polyester (PermaNet 2.0) or electrostatic gauze with two concentrations of deltamethrin

			PermaNet 2.0, DM 55 mg AI/m^2			Electrostatic gauze, DM 3.7 mg AI/m^2			Electrostatic gauze, DM 37 mg AI/m^2		
Mosquito	Strain	Status	*N*	Knockdown, % (95% CI)	Mortality, % (95% CI)	*N*	Knockdown, % (95% CI)	Mortality, % (95% CI)	*N*	Knockdown, % (95% CI)	Mortality, % (95% CI)
Anopheles gambiae	Kisumu	Susceptible	40	97.5 (92.6–100)	100	42	100	100	40	100	100
	SUA	Susceptible	54	100	100	51	100	100	55	100	100
	Tiassale	Resistant	47	70.6 (62.0–79.1)	9.6 (1.2–18.0)	47	93.2 (86.3–100)	100	49	97.9 (94.0–100)	100
	Tongs	Resistant	55	60.0 (47.0–72.9)	58.2 (24.9–51.1)	46	95.7 (89.8–100)	95.7 (89.8–100)	57	100	100
Anopheles arabiensis	KBG	Susceptible	45	100	100	52	100	100	53	100	100
	SENN_DDT	Resistant	52	88.5 (79.9–97.1)	76.9 (65.5–88.3)	45	75.6 (63.1–88.1)	82.2 (71.0–93.4)	55	100	100
	MBN-DDT	Resistant	48	12.5 (3.1–21.9)	37.5 (23.8–51.2)	53	88.7 (80.1–97.3)	96.2 (91.1–100)	54	100	100
Anopheles funestus	FANG	Susceptible	48	100	100	53	100	100	52	100	100
	Fumoz-R	Resistant	47	8.5 (0.5–16.5)	10.6 (1.8–19.4)	46	17.4 (6.4–23.4)	39.1 (25.0–53.1)	46	95.7 (89.8–100)	63.0 (49.1–76.9)
	Fumoz-base	Resistant	51	5.9 (0.0–12.4)	7.8 (0.4–15.2)	51	13.7 (4.3–23.1)	49.0 (35.3–62.7)	53	92.5 (88.4–99.6)	83.0 (72.8–93.2)
Culex quinquefasciatus	I2C-CX	Unknown	30	43.3 (25.7–60.9)	40.0 (22.6–57.4)	50	10.0 (1.8–18.2)	82.0 (71.4–92.6)	31	93.5 (86.4–100)	100
	Wild-type	Unknown	41	2.4 (0.0–7.1)	4.9 (0.0–11.6)	48	52.1 (38.0–66.2)	95.8 (90.1–100)	36	56.5 (42.2–70.8)	93.5 (86.4–100)
Aedes aegypti	I2C-AE	Unknown	32	84.4 (71.9–96.9)	87.5 (76.1–98.9)	52	73.1 (61.0–85.3)	92.3 (85.1–99.6)	50	100	100

Knockdown and mortality rates are shown with 95% CIs calculated for each pooled sample proportion. DM, deltamethrin.

Table S2. Corrected knockdown and mortality as a percentage of the total number of exposed mosquitoes after 5-s contact exposure to PermaNet 2.0 polyester coated with deltamethrin and deltamethrin-dusted electrostatic gauze with two concentrations of active ingredient

			PermaNet 2.0, DM 55 mg AI/m^2			Electrostatic gauze, DM 3.7 mg AI/m^2			Electrostatic gauze, DM 37 mg AI/m^2		
Mosquito	Strain	Status	*N*	Knockdown, % (95% CI)	Mortality, % (95% CI)	*N*	Knockdown, % (95% CI)	Mortality, % (95% CI)	*N*	Knockdown, % (95% CI)	Mortality, % (95% CI)
Anopheles gambiae	Kisumu	Susceptible	48	100	100	42	100	100	40	100	100
	Tiassale	Resistant	47	63.8 (50.1–76.3)	6.4 (0.0–13.5)	51	15.7 (5.7–25.7)	37.3 (24.0–50.6)	50	96.0 (90.5–100)	82.0 (71.4–92.6)
Culex quinquefasciatus	I2C-CX	Unknown	39	20.7 (6.0–35.4)	20.7 (6.0–35.4)	38	2.6	50.0 (34.1–65.9)	38	57.1 (38.7–75.5)	78.6 (63.3–93.9)
	Wild-type	Unknown	50	0.0	40.0 (26.5–53.5)	50	4.0	60.0 (46.5–73.5)	50	38.0 (24.5–51.5)	60.0 (46.5–73.5)
Aedes aegypti	I2C-AE	Unknown	42	31.0	31.0 (17.1–44.9)	45	46.7	62.2 (48.1–76.3)	50	100	100

Knockdown and mortality rates are shown with 95% confidence intervals calculated for each pooled sample proportion. DM, deltamethrin.

Table S3. Origin, rearing, strain, and exposure specifics of the susceptible mosquitoes and the mosquito strains with undefined resistance used in the insecticide bioassays

Mosquito species	Susceptible mosquitoes				Mosquitoes with undefined resistance		
Mosquito species	*An. gambiae s.s.*	*An. gambiae s.s.*	*An. arabiensis*	*An. funestus*	*Ae. aegypti*	*Cx. quinquefasciatus*	*Cx. quinquefasciatus*
Mosquito strain	Kisumu	SUA	KGB	FANG	I2C-AE	I2C-CX	Field-collected
Origin	Kisumu, Kenya	Suakoko, Liberia	Kanyembe, Zimbabwe	Southern Angola	Aruba	USA	Kilombero, Tanzania
Reared by	LSTM (LITE)	VCRU	VCRU	VCRU	In2Care	In2Care	N/A
Selected resistance	None	None	None	None	None (field-collected 01/2012)	None	None (field-collected 03/2014)
Resistance profile	Fully susceptible	Fully susceptible	Fully susceptible	Fully susceptible	Unknown	Fully susceptible	Fully susceptible
Exposure date	13/03/14	28/11/14	26/11/14	30/11/14	10/02/14	17/02/14	28/03/14
Mosquito age	3–6 d	2–4 d	2 d	2 d	3–6 d	3–4 d	2 d

LST (LITE), Liverpool School of Tropical Medicine (Liverpool Insect Testing Establishment).

Table S4. Origin, rearing, strain, resistance status, and exposure specifics of mosquito strains with well-defined resistance profiles used in the insecticide bioassays

Mosquito species	*An. gambiae s.s.*	*An. gambiae s.s.*	*An. arabiensis*	*An. arabiensis*	*An. funestus*	*An. funestus*
Mosquito strain	Tiassale	Tongs	SENN-DDT	MBN-DDT	Fumoz-R	Fumoz-base
Origin	Tiassale, Burkina Faso	Tongon, Ivory Coast	Sennar, Sudan	KwaZulu Natal, South Africa	Mozambique	Mozambique
Reared by	LSTM (LITE)	VCRU	VCRU	VCRU	VCRU	VCRU
Selected resistance	Pyrethroid resistance	Multiple, no selection since 2010	DDT resistance (selected since 1995)	DDT resistance (selected until 2013)	Permethrin resistance (selected until 2001)	Naturally multiple resistant
Resistance profile	Kdr and P450s	Not known	Kdr, GSTs, P450s, esterases	Kdr, P450s, GSTs, esterases	P450s, GST	P450, GST
Exposure date	13/03/2014	26/11/14	25/11/14	01/12/2014	25/11/14	27/11/14
Mosquito age	2–4 d	3 d	3 d	2 or 3 d	2–4 d	2–4 d

LST (LITE), Liverpool School of Tropical Medicine (Liverpool Insect Testing Establishment).

PLOS | NEGLECTED TROPICAL DISEASES

POLICY PLATFORM

Quantifying the Epidemiological Impact of Vector Control on Dengue

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Introduction

Dengue virus (DENV) is a self-limiting illness in tropical and subtropical regions around the globe caused by four closely related, but distinct, virus serotypes (DENV-1, -2, -3, and -4) that are transmitted among humans by mosquitoes, primarily *Aedes aegypti* [1]. Approximately 4 billion people living in more than 128 countries are at risk of infection [2]. Each year there are an estimated 400 million new infections, of which about 100 million manifest as apparent illness [3]. The outcome of human infections ranges from asymptomatic to mild illness to severe, life-threatening disease [4]. DENV not only causes more human morbidity and mortality than any other arthropod-borne virus but it is also a growing public health threat. There has been a dramatic 4-fold increase in dengue cases between 1990–2013 and dengue continues to expand in geographic range [2,3,5,6].

Presently, vector control is the primary means for preventing dengue [7]. Several vaccine constructs are in clinical trials and initial results are encouraging [8]; recently licensure was

granted for the Sanofi Pasteur vaccine in Mexico, Brazil, and the Philippines [9]. A few well-documented successes indicate that, when rigorously applied, vector control can reduce dengue. The advent of DDT in 1947 led to a hemisphere-wide program in the 1950s and 1960s across Central and South America that dramatically reduced *Ae. aegypti* populations, resulting in impressive reductions in yellow fever and dengue [10]. During the 1970s–1980s [11] and the 1980s–1990s [12], respectively, Singapore and Cuba successfully used vector control and larval source reduction to reduce the force of DENV infection (i.e., per capita risk of human infection [13]) and, thus, disease. Recent trials of indoor residual spraying [14] and indoor space spraying [15] appeared to reduce human DENV infections. Regrettably, these control achievements were rare and ultimately transient. Dengue reinvaded Latin America after the *Ae. aegypti* eradication campaign ended, rebounded in Singapore and Cuba after 20 and 16 years of successful control, respectively, and is increasingly being reported in Africa due to improved surveillance [16].

Although the concept of dengue vector control seems straightforward, successful broad-scale application has been difficult to achieve and even harder to sustain [17]. In most settings, dengue vector control failed to prevent epidemics and it is not slowing expansion of the virus's geographic range [3,17,18]. Unsuccessful control programs are often attributed to inadequate responses to a robust virus transmission system. Outbreaks may occur due to combinations of risk factors, including expanding *Ae. aegypti* populations, virus and mosquito dispersal via extensive human travel networks, weak vector control infrastructure, lack of resources to mount effective interventions, lack of political will, and ineffective implementation of existing tools and strategies [17,19]. A recent review concluded that dengue vector control can be effective, but only when implementation is expedient, comprehensive, and sustained [7].

Despite these major challenges, there is growing interest in combining vector control with vaccination once a dengue vaccine becomes widely available, which recognizes that one intervention is insufficient to effectively reduce the burden of disease. Theoretically, a dengue vaccine could elevate herd immunity, making it easier to sustain the effects of vector control on virus transmission. Similarly, vector control could lower the force of DENV infection, making it easier to achieve vaccine delivery goals [20]. Results from studies with malaria [21,22] and lymphatic filariasis [23] support the impact of simultaneously targeting vectors and pathogens.

The next critical step is selecting vector control strategies that are best suited for combining with a vaccine. Selection criteria will likely depend on local dengue ecologies. Some funding agencies are responding by enabling investigators and developers to carry out quantitative and epidemiologic assessments of novel approaches, e.g., release of *Wolbachia*-infected *Ae. aegypti* [24,25], spatial repellents and vapor active insecticides [26,27], and enhanced community mobilization [28] as part of early-phase intervention evaluations [24]. Surprisingly, most existing dengue vector control strategies (e.g., larvicides and outdoor versus indoor insecticide space spraying) have not been robustly evaluated for impact on reducing human infection and disease [29,30]. Some trials have evaluated entomological impact [31], but reductions in mosquito populations do not correlate well with predictable reductions in dengue disease [20,32]. Along with underpowered and inefficient control responses, the fact that current dengue vector control tools and strategies lack quantitative evidence of efficacy from field trials helps explain why contemporary control programs fail more often than they succeed.

A Partnership for Dengue Control-sponsored workshop was convened to begin to address this gap [33]. A panel of international experts identified the vector control tools currently available that may have the highest probability of success in reducing dengue and field trial experimental design attributes necessary to assess their efficacy. Although vaccines will likely be implemented concurrently with vector control, before that can be done epidemiological trials are needed to quantify the protective efficacy of vector control interventions alone. Results will provide a benchmark for subsequent trials in which combinations of interventions are assessed.

This approach builds on recent calls for increased rigor in the design of vector control studies [34] with an emphasis on dengue. In addition to benefiting dengue control programs, the results of the proposed trial described herein will be relevant for prevention of other *Ae. aegypti*-borne viral infections of increasing public health importance, such as chikungunya [35] and Zika [36] viruses.

Dengue Vector Control Experimental Design Considerations

Ecological Complexities

Aedes aegypti will be the primary target of our intervention. Other mosquito species such as *Aedes albopictus* and *Aedes polynesiensis* can play secondary roles in DENV transmission in specific geographic areas [37], but a trial site where *Ae. aegypti* is the only vector species present is desirable to simplify interpretation of trial results. Given the overlapping distribution of *Ae. aegypti* and other vectors of DENV, especially *Ae. albopictus*, if this is not achievable, the difference in ecology across the species present must be taken into account when selecting control methods, intervention application locations, and in the interpretation of results. If a site where only *Ae. aegypti* is present is identified, trials may need to be repeated where other vector species are present.

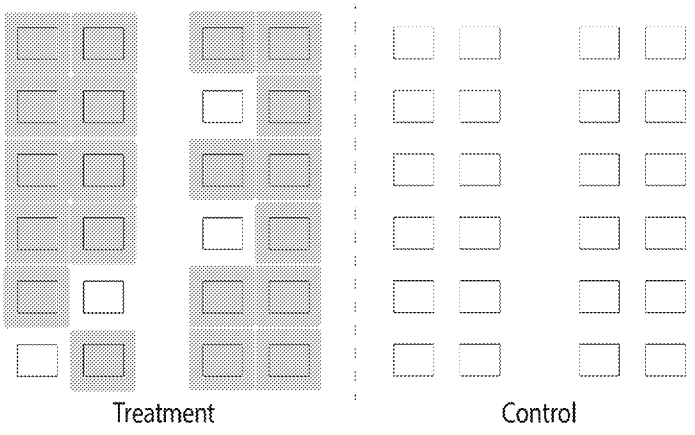
DENV transmission is local, focal, heterogeneous, and highly efficient [38,39]. Transmission is facilitated by *Ae. aegypti*, which live in relatively low densities in close association with humans and seldom fly farther than 100 m [40]. Transmission foci are connected at short distances by a combination of human and mosquito movement patterns and at longer distances by human movement alone [38,41]. Clusters of mosquitoes and the houses they infest (high-density hotspots) are highly focal, seldom larger than 30 m, and, even though they can consistently be detected, they are temporally unstable, i.e., fine-scale mosquito abundance is continually shifting across time and space [42]. Because *Ae. aegypti* is a day-biting mosquito, people are at risk of infection both in their homes and when they leave home. We therefore recommend (1) accounting for human movement in a cluster-randomized controlled trial (RCT) study design by either applying means to measure movement patterns during the follow-up period or focusing on a least mobile section of the population and (2) conducting intervention trials in areas with historically high levels of human infection rather than attempting to identify and attack mosquito hotspots.

Possible Control Tools

Given *Ae. aegypti*'s peri-domestic habits, key candidate tools for vector control are those based on the use of long-lasting formulations of synthetic pyrethroids applied to walls, curtains, window screens, and water container covers [7]. Reduction of larval sources through either container removal or applications of insecticides or biological agents can decrease adult mosquito production [31], but mosquitoes often exploit cryptic containers that remain untreated without extensive insecticide fogging. To be most effective, larval control needs to be combined with methods targeting adult mosquitoes [7]. Recent evidence suggests that insecticide-treated curtains, window screens, and covers on water-holding containers can reduce *Ae. aegypti* densities in and around treated areas [43–45], indicating that these tools should be considered in an integrated approach. Innovative vector control tools that are currently being evaluated for malaria should be explored for dengue.

Defining Impact

The protective efficacy (PE) of a treatment relates the risk of infection within a group that receives the treatment to that of an untreated control group (see Fig 1). During the initiation of



Consider two blocks under treatment and two under control. If every home participates in the treatment, the *protective efficacy*, PE, is given as:

$$PE = 1 - \frac{\text{Risk of infection in treatment group}}{\text{Risk of infection in control group}}$$

If f represents the percent of individuals accepting intervention (i.e., coverage level), then the direct, indirect, total, and overall protective efficacy (PE_D, PE_{ID}, PE_T, and PE_O) are given as [adapted from [59]]:

$$PE_D = 1 - \frac{\text{Risk in accepting treatment homes}}{\text{Risk in non-accepting treatment homes}}$$

$$PE_{ID} = 1 - \frac{\text{Risk in non-accepting treatment homes}}{\text{Risk in control homes}}$$

$$PE_T = 1 - \frac{\text{Risk in accepting treatment homes}}{\text{Risk in control homes}}$$

$$PE_O = 1 - [f(1 - PE_T) + (1 - f)(1 - PE_{ID})]$$

Fig 1. Protective efficacy: basic definitions.

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the trial, special messaging will be used to maximize the participation rate. By accounting accurately for individuals (or homes) within treatment areas that decline to participate in the trial, but still provide data on infection status, the relative direct and indirect effects of the trial can be calculated (Fig 1). Classical models originally developed for malaria [46] and subsequently applied to other mosquito-borne pathogens [47] offer a range of metrics for intervention trials (e.g., vectorial capacity, entomological inoculation rate, and basic reproductive number [48]) and a means for mathematically connecting those metrics with other measurable outcomes (e.g., attack rates and the force of infection [49]). These relatively simple mathematical associations offer an effective way to explore the expected relative impacts of an intervention with a given metric, e.g., a 50% reduction in larval habitats can lead to a 75% reduction in vectorial capacity [50]. After empirically-driven estimates of the impact of control strategies have been calculated from an RCT, the mathematical form of these dynamics will help identify metrics most likely to track transmission intensity and support the recommendation of appropriate combinations of interventions to achieve maximum reduction in infection and disease.

Measuring Outcomes

Vector control is designed to directly impact mosquito populations. Although the primary endpoints of the proposed study are epidemiological, there are several crucial reasons why mosquito populations should be measured. In addition to continual testing for insecticide resistance in adults and larvae, longitudinal monitoring of immature and adult mosquito densities are necessary to confirm that the intervention affected the mosquito population as expected, and adult mosquito data can be used to assess the relative transmission risks inherent in each cluster. If mosquito densities vary in space so that some locations systematically over-

or under-produce compared to other locations, population dynamics should be characterized before the trial begins and used to stratify randomization of treatment and control clusters.

Epidemiologic impact will be determined using three complementary approaches: longitudinal cohorts, febrile surveillance, and geographic clusters. Seroconversion by participants in prospective longitudinal cohorts is one way to accurately detect differences in human DENV infection and relative risk of infection [24,51]. Identifying seroconversions to tertiary or quaternary infections is difficult, thus a pediatric cohort in endemic settings would be advantageous to maximize transmission detection (i.e., people who enter the study as immunologically naïve or have a monotypic antibody response) and minimize the potential for movement between treatment and control study arms. Serostatus will be monitored to determine if there was more than one infection between any two tests. At locations where more than one flavivirus is transmitted (e.g., yellow fever, Japanese encephalitis, Zika) or where people have been vaccinated against other flaviviruses (e.g., yellow fever, Japanese encephalitis), interpretation of DENV seroconversions will need to account for cross-immunity to closely related viruses. If a trial is conducted in concert with, or soon after, administration of a DENV vaccine, the design can include a surrogate endpoint of clinically apparent DENV infections that are virologically confirmed, similar to what has been done in recent dengue vaccine trials [52]. A serologic-based endpoint could be used for vaccinated populations if, in the future, new assays are developed that can differentiate the immune response to vaccination versus a natural DENV exposure.

Within a subset of identified neighborhood clusters, participants will be recruited for a longitudinal cohort. Routine febrile surveillance consisting of one to three visits per household per week of people living near cohort participants will allow longitudinal comparisons of people with documented dengue illness [38]. Geographic cluster studies that screen people living within a designated radius (~100m) of a person with a laboratory-diagnosed DENV infection (the index case) will measure variation in fine-scale spatial patterns of DENV transmission [24]. Depending on how movement is accounted for or incorporated into the trial design, cohort sample size estimates will be in the range of 2,000 to 3,000 participants [51], with at least five times that number under febrile surveillance [38]. The number of clusters in the study will depend on background transmission, local herd immunity, anticipated effect size of the intervention, between cluster variation, and logistics capacity.

Design and Analysis

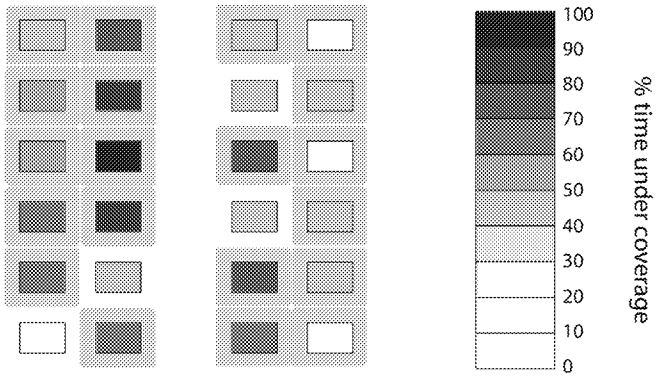
Given the diffusive effect of mosquito dispersal on transmission risk, vector control trials typically employ a cluster RCT study design [53] in which communities are randomly allocated to intervention or control arms [34]. One of the largest challenges with measuring the impact of vector control on dengue transmission, as opposed to other mosquito-borne diseases such as malaria, is that dengue vectors bite during the day. People who live within a cluster assigned to receive the intervention may, therefore, spend a considerable amount of their day at risk of infection in untreated areas. Conversely, those living in untreated areas may move into treated areas during their daytime activities. To estimate accurately the effectiveness of vector control on dengue transmission, information on the movement patterns of individuals within the treatment and control arms is needed to estimate individual-level time under coverage. One way to limit the effect of human movement is to have clusters that include large geographic areas and to enroll children who may not be as mobile as adults, although this may be operationally difficult to achieve and expensive. The effectiveness of a community-applied strategy, as often occurs in vector control, will likely only be fully felt by those who never leave the treated area (see Fig 2). As an individual spends increasingly more time in locations where transmission continues unhindered, their individual risk of infection recovers to background levels. By incorporating

Measuring movement / time under coverage

GIS/diffusion: Using exact locations for each home, and an estimated diffusion kernel, simply integrate the kernel over the treated area centered at the home of each participant.

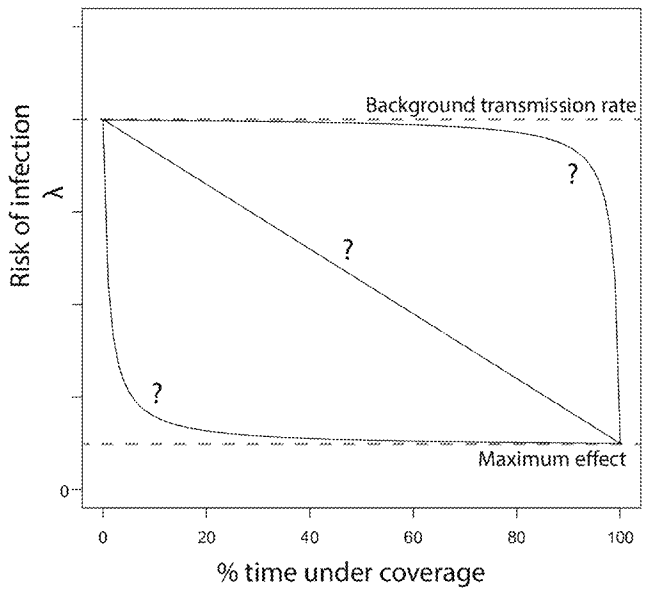
Movement surveys: Using a sequence of retrospective movement surveys, parameterize a movement algorithm for each individual. Sum up estimated time spent within treated area.

GPS: Track the location of each (or a sample) of participants and sum time within treated area.



Individuals living in the interior of the treatment area will likely spend more time under coverage in the treated area. Individuals spend a considerable amount of time in their home, so non-participating individuals living on the edge may experience minimal protective effect.

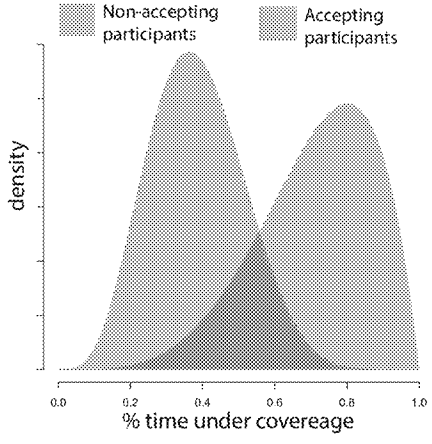
Measuring efficacy



Measuring individual movements results in the ability to fit the relationship between 'time under coverage' and risk of infection. Even if no individuals remain under coverage continuously the entire trial, the maximum effect may be estimable. Different clusters can be included as random effects.

The shape of this relationship may vary from one context to another and should be fit with a flexible functional form (e.g., splines).

Calculating protective efficacy



Under the simplifying assumption that individuals in the control area never visit a treatment area (an assumption easily removed), calculating protective efficacy requires appropriately weighting the observed data and fitted relationship between time under coverage and risk of infection. If p_A represents the pdf of time under coverage for individuals who participate and live in the treatment area, p_N represents the pdf of time under coverage for individuals who refuse to participate, but live in the treatment area, and $\lambda(t)$ is the fitted relationship between time under coverage and risk of infection, then we can redefine protective efficacy as:

$$PE_D = 1 - \frac{\int_0^1 p_A(t)\lambda(t)dt}{\int_0^1 p_N(t)\lambda(t)dt} \qquad PE_{ID} = 1 - \frac{\int_0^1 p_A(t)\lambda(t)dt}{\text{Risk in control homes}}$$

$$PE_T = 1 - \frac{\int_0^1 p_A(t)\lambda(t)dt}{\text{Risk in control homes}} \qquad PE_O = 1 - [f\,(1 - PE_T) + (1 - f)\,(1 - PE_{ID})]$$

Fig 2. Estimating protective efficacy when considering movement out of coverage areas.

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individual-level time under coverage in analyses, the maximum possible effect (as well as the average predicted effects) of the intervention can be estimated (see Fig 2). When possible, if movement patterns systematically differ across cluster arms, random assignment of clusters to treatment versus control should be stratified by movement level, much like cluster stratification using baseline incidence or prevalence rates commonly employed in malaria trials [54].

An alternate design to the "dose-response" approach described above would focus on only those individuals who move the least. Initial movement surveys may identify a sub-population that is less mobile, i.e., young children. Clusters can then be defined based on movement range and design, and analysis can use the standard metrics described in Fig 1. This approach might necessitate large numbers of participants in clusters if fewer susceptible individuals qualify for enrollment. On the other hand, this approach directly assesses treatment with a more rigorous, but potentially less valid, assumption of equal exposure to all those who are treated. The choice as to which study design is optimal will likely be context and resource dependent. If the more rigorous option is taken, movement data and analyses metrics in Fig 2 can be applied as a secondary endpoint.

Site Selection

Trial site selection will follow a detailed process of criteria evaluation to identify locations with the capacity to rigorously assess the intervention [34]. DENV must be endemic with existing information on transmission intensity and local *Ae. aegypti* population dynamics. Infrastructure and trained personnel must be in place for measuring entomological and epidemiological outcomes. Community engagement of study participants should be ongoing throughout the entire trial period. Public opinion and compliance will be key for a successful assessment. A framework for communicating with stakeholders has been developed for evaluating genetically modified mosquitoes, a strategy in which public opinion and compliance are challenging and from which trials using other vector control interventions could benefit [55].

Preliminary studies on opinions about vector control will help inform the most effective strategy. Local *Ae. aegypti* populations will be screened for resistance to determine available insecticides for each potential site. Baseline virology and vector sampling should be carried out for one year before interventions and intervention should last for at least two transmission seasons to account for inter-annual variation in transmission and mosquito population densities. It will be important for funders to understand the return on investment of longer duration trials for informed decision-making.

Concerns

Insecticide Resistance

Insecticide resistance is a particularly worrisome challenge for dengue prevention because most dengue vector control strategies rely heavily on the use of chemical control. The resistance status of the target vector population should be taken into consideration when designing interventions to ensure that the product used will not only achieve maximum impact but have sustained effects throughout anticipated DENV transmission cycles. Resistance should be monitored throughout the trial, e.g., by knockdown resistance (kdr) and WHO bioassays [56]. Given the extended duration of continual insecticide application, options to mitigate selection pressure should be determined prior to intervention roll-out to avoid, mitigate, and minimize the development of resistance associated with the specific interventions. The degree to which insecticide resistance compromises dengue vector control efficacy remains largely unknown, but should not be underestimated given that the most cost-effective vector control tools rely heavily on the use of a highly limited number of public health insecticides [57].

Ethics

The ethical, social, and cultural framework for trial site selection outlined by Lavery et al. [58] will be applied. As such, community authorization and participation is a priority for site selection and conducting a meaningful trial. After the regulatory process has been defined, thorough ethical evaluation of the study design will be carried out, including the possible use of a placebo if no established intervention is used. During the trial, government and public health dengue control programs must be allowed to conduct routine vector control activities because this cannot ethically be prevented or hindered. Trial management should follow fundamentals used for Good Clinical Practice, including standard operating procedures; a trial steering committee; convening or engaging data monitoring, safety, and ethics committees; a dedicated trial monitor; and that the analytical plan should be finalized before commencement of the trial.

Recommendation

The "local and focal" nature of DENV transmission and the often large variation in transmission patterns from year to year, combined with the short-range dispersal of the day-biting vector *Ae. aegypti*, create a challenging context for evaluating the effectiveness of any dengue vector control intervention. Detecting a causal relationship between entomological impact and a reduction in dengue transmission intensity requires careful trial design. Within the design of an RCT, it is important to account for human movement patterns. If human movement is ignored, a successful intervention may appear to have failed. Ignoring human movement during study design considerations would require an artificial increase in the expected effectiveness to achieve adequate power because every individual in a treatment cluster will be predicted to have the "maximum" effect. Ignoring human movement during analysis would almost certainly reduce the ability to detect a significant effect of the intervention due to spillover of "treated" individuals into areas without protection. Potential confounding factors such as the development of insecticide resistance over the course of the trial or an unanticipated lack of residual effect of the intervention could directly compromise trial outcomes and should be closely monitored. Building a bridge between dengue control experiences and those of other vector-borne pathogens, especially *Plasmodium*, may accelerate advancement of control techniques across multiple diseases.

Conclusions

Very few vector control tools for any mosquito-borne pathogen have been assessed in Cochrane-style reviews, which are generally considered the most rigorous assessment of health interventions, largely due to design flaws [34]. For dengue, there are very few published studies [28] and even fewer studies that formally assess the impact of existing insecticide-based strategies on dengue. Although there is progress in the development of a dengue vaccine, it is likely for the foreseeable future that integrating vector control and vaccination will be necessary [33]. Integrated Vector Management is endorsed by the World Health Organization for the control of dengue and other vector-borne diseases [57]. Insecticides cannot be distributed and disseminated in vast quantities across broad geographic areas without concern for ecological and environmental impact. Subjecting any population to continued exposure to insecticides requires precise estimates of both the costs (economic and environmental) and the public health benefits.

As dengue's global burden grows, the need for proven effective vector control options will increase. We argue that quantifying the epidemiological impact of any vector control intervention on DENV transmission will require assessments of human movement. We propose two options: cluster sizes can be enlarged and maximum age of participants can be reduced until protection is approximately uniformly felt by all those who are intended to be treated. This

may, however, greatly increase required resources for already economically challenging trials. The alternative option is more efficient, but comes with some cost in rigor. As described in Fig 2, human movement patterns can be explicitly incorporated into the calculations for protective efficacy, with movement either inferred from simple geographic-based movement kernels or explicitly estimated by extrapolating the movement patterns of a sub-population within each cluster. Using either approach, intervention trials can provide robust and meaningful information. Insights from such trials will help guide the scaling up of effective dengue control strategies, whether vector control alone or in combination with vaccines, and will be applicable to other *Ae. aegypti*-borne viral infections of current public health concern, such as chikungunya and Zika viruses.

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PLOS | NEGLECTED TROPICAL DISEASES

Viewpoints

Considerations in the Design of Clinical Trials to Test Novel Entomological Approaches to Dengue Control

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Introduction

Dengue is the most important arboviral infection of humans. In endemic countries the scale of the dengue disease burden imparts an economic cost [1] and strains fragile health care systems. There are no licensed vaccines for prevention of dengue, and the public health response in endemic countries relies mostly on combating the principal mosquito vector, *Aedes aegypti*, via insecticides and breeding site removal. The sustained transmission of dengue in endemic settings together with its increasing global footprint indicates existing disease control strategies have been unsuccessful [2].

Novel vector control approaches to limit dengue virus (DENV) transmission include release of *Ae. aegypti* that carry transgenes that result in highly penetrant, dominant, late-acting, female-specific lethality [3]. In field cage experiments, the release of such mosquitoes in sufficient numbers results in eradication of the mosquito population [4]. Another strategy involves embryonic introduction of the obligate intracellular insect bacterium, *Wolbachia*, into strains of *Ae. aegypti* [5]. Strikingly, *Wolbachia*-infected *Ae. aegypti* are partially resistant to infection with DENV [6], and by virtue of the intrinsic capacity of some strains of *Wolbachia* to invade insect populations [6,7], there is the prospect of achieving widespread biological resistance to DENV amongst *Ae. aegypti* populations. The life-shortening impact of some Wolbachia strains could also contribute to reductions in disease transmission [5]. The first entomological field trials of mosquitoes infected with *Wolbachia* (wMel and wMelPop strains) have now been successfully carried out in Cairns, Australia and have demonstrated that *Wolbachia* can establish itself at very high prevalence in field populations of *Ae. aegypti* [7]. However, the prospects of demonstrating reduction in DENV transmission in Cairns are slim given the episodic, imported nature of dengue outbreaks in this region.

A critical challenge for all entomological approaches to control of vector-borne disease is how best to demonstrate efficacy in reducing disease transmission [8]. In principal, the high force of infection in dengue endemic countries should assist an evidence-gathering approach to this challenge. However, a feature of dengue epidemiology is that it is spatially and temporally heterogeneous [9–11]. Thus oscillations in disease incidence over time are common for a given region of transmission, and within each region it is common for focal "hot spots" of transmission to exist [3]. This heterogeneity in transmission means that uncontrolled observational studies of dengue transmission in a community where, for example, *Wolbachia*-infected *Ae. aegypti* have been released could take many years or decades to yield evidence that is suggestive of a benefit. Equally, the heterogeneity of dengue transmission poses challenges to traditional clinical trial approaches, as does the non-stationary nature of mosquito populations [8]. Here we review design and statistical considerations relevant to the conduct of clinical trials of these novel interventions and the practical challenges posed by the epidemiology of dengue in endemic settings. Whilst our discussion of trial design is focused on *Wolbachia*-infected *Ae. aegypti*, it is also relevant to other vector control interventions, such as genetically engineered male mosquitoes carrying a dominant lethal gene [4], insecticide-impregnated nets [12], or larvacides [13].

Methods

Cluster randomised trials (CRTs) are the gold standard design to provide evidence on the efficacy of an intervention that has community-wide impact [14]. Cluster formation is a crucial aspect of the design of a CRT and requires prior mapping of the study area with respect to dengue sero-prevalence, demographics, and information on movement of individuals. Experience from the Cairns (Australia) release shows that it is feasible to achieve a prevalence of Wolbachia infection in *A. aegypti* mosquitoes of nearly 100% in treatment clusters within 6 months after first release [7]. Clusters need to be sufficiently geographically separated to ensure that *A. aegypti* mosquitoes present in control clusters remain virtually free of *Wolbachia* for the entire study period.

We consider the incidence of DENV-seroconversions during a trial as a suitable primary endpoint and DENV-naïve children aged 2–5 years living in each cluster as an optimal "sentinel" cohort for serological surveillance. Young children are less likely to spend substantial periods of time outside of their residence and local community (and hence outside of the "treatment umbrella") than more mobile older children and adults. In addition, DENV-prevalence in older children is higher and those remaining naïve and

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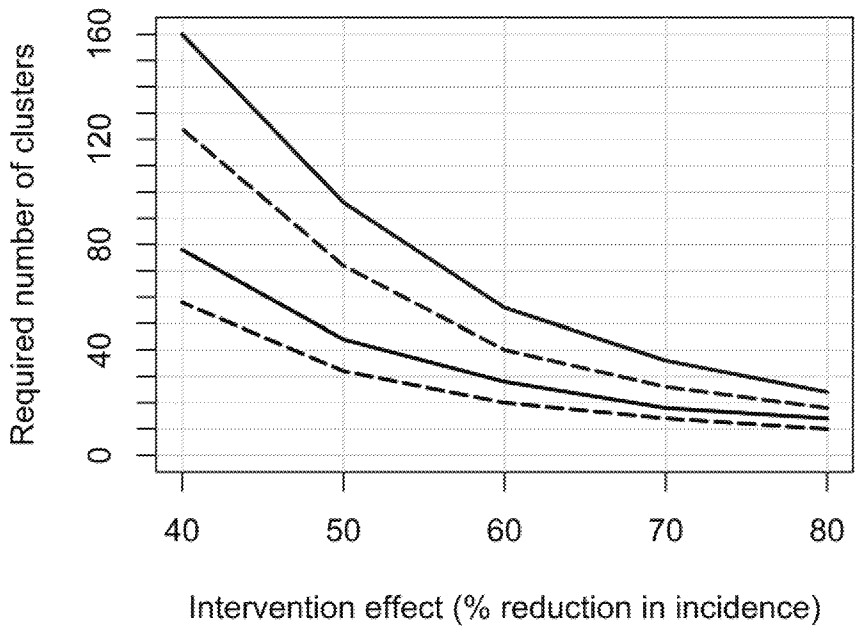

Figure 1. Sample size estimates for a PCRT or a SWCRT. Total number of clusters required for a PCRT (black lines) or a SWCRT (blue lines) depending on the size of the intervention effect. Solid lines correspond to 90% power, dashed lines to 80% power. Simulations are based on parameters determined from the Kamphaeng Phet dengue cohort (Thailand) (described in [10]) with three time periods each of 1-year duration, a surveillance cohort of 100 children in each cluster, and a two-sided significance level of 5%.
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hence eligible for the study are potentially less representative of the full population (for example, for socio-economic reasons).

Two alternative designs are considered. The first is the classical parallel two-armed cluster randomised trial (PCRT) in which each recruited cluster is randomised to intervention or control, and the intervention is implemented simultaneously across the relevant clusters. Thus the control clusters provide contemporaneous controls for the intervention clusters. The other design considered is a stepped wedge cluster randomised trial (SWCRT) in which each cluster is assigned to the control treatment initially and clusters are subsequently crossed-over to the intervention in a random selection at fixed time points until eventually all clusters are under treatment [15,16]. As dengue is a seasonal disease, selected cross-over time points should reflect this. As an example, for a 3-year study period, the SWCRT has: all clusters as controls for year 1; half of the clusters as controls and half as intervention, randomly selected, for year 2; and all clusters on intervention in year 3. Diagrams of both designs are provided in Text S1.

SWCRTs have been most frequently used for evaluating interventions during routine implementation such as the evaluation of a vaccine on the community level following a successful individual randomised trial. From a logistic perspective, they are attractive, because the intervention can be rolled out in a step-wise fashion and evaluated. As clusters are their own controls, SWCRTs are less sensitive to between-cluster variation and thus might require a lower sample size compared to parallel designs [15]. However, strong temporal effects may greatly reduce the precision of estimates as all clusters start out in the control arm and end as intervention clusters. Secular trends of dengue during the study period could confound the treatment effect causing bias. SWCRTs are less flexible for trial adaptations such as an extension of the follow-up period if the observed DENV-incidence is lower than expected, as all clusters have already crossed-over to the intervention at this time point.

Cluster size and cluster separation are important considerations in the design of all CRTs, but they require particular attention in trials of vector control interventions, for which entomological and community considerations need be taken into account. Entomological considerations include the dispersal of *Wolbachia*-infected mosquitoes to ensure a persistent and homogenous effect in treatment clusters without undue contamination into untreated clusters that serve as controls. For dengue trials community considerations include the extent of daily movement within and between clusters that the surveillance cohorts are likely to undertake; if the clusters are too small this movement may be excessive, and cause further reduction in any treatment effect. Thus, data on movement patterns of children eligible to join the surveillance cohort together with more information on the limits of spatial dispersal of *Wolbachia*-infected mosquitoes are essential before the cluster formation stage of any trial. An approach that is widely adopted in CRTs is the so-called "fried-egg" design [14], in which the whole cluster receives the allocated treatment but only the inner area of the cluster (the "egg-yolk") is used for surveillance since the treatment effect in this inner area is less affected by spill-over from neighbouring clusters that may be in the opposite treatment arm. We would therefore suggest that the surveillance cohort in each cluster be drawn from this inner area of each cluster.

Sample Size Requirements of a CRT

Sample size requirements for CRTs of a *Wolbachia* intervention (or other community-based intervention) depend critically on the size of the intervention effect and on both the magnitude and the variability (temporal and spatial) of seroconversion rates between clusters. To assess this variability in an example, we used published data from 12 primary schools in Kamphaeng Phet, Thailand, followed over a 3-year period [10] where the overall

yearly DENV infection incidences were 7.9%, 6.5%, and 2.2%.

A mixed-effects Poisson-regression model fitted to these data gave coefficients of variation (cv, i.e., SD/mean) for yearly DENV infection incidence of 0.27 for between-school variation, 0.57 for annual variation, and 0.85 for residual variation (i.e., variation that cannot be explained by systematic spatial or temporal variation, respectively, and corresponds to localized school and year specific variation). A detailed description of the model used to derive these coefficients of variation can be found in Text S1. The overall between-school coefficient of variation over the 3-year period was 0.52. The same model fit to data from 43 villages in Cambodia [9], also showed that temporal and residual variation are more pronounced than spatial variation (unpublished data).

We then used the incidence and variability data reported above to simulate hypothetical PCRT and SWCRT trials. Additional assumptions for the trial simulations were a study duration of 3 years and a surveillance cohort of 100 children in each cluster. We varied the intervention effect between a 40% and an 80% decrease of DENV seroconversion in intervention clusters compared to controls. Allowing for the fact that some children in intervention clusters will experience infections outside of the intervention area, we regard an effect of a 50%–60% reduction as realistic in our target population. Details regarding the set-up of the simulation study and the statistical analysis of simulated trials are provided in Text S1.

Results

Sample size requirements for the two designs and for varying treatment effects are shown in Figure 1 and requirements for several alternative scenarios are given in Text S1. The required total sample sizes to detect a 60% or 50% reduction of dengue in the intervention arm with 80% power were 20 or 32 clusters, respectively, for a PCRT compared to 40 or 72 clusters for a SWCRT. The SWCRT design generally required substantially higher sample sizes except in the unrealistic situation of spatial but no temporal or residual variation.

Conclusions

A parallel cluster-randomised trial is the design of choice for testing novel entomological methods of dengue control. Under realistic assumptions we show it to require a substantially lower sample size than a stepped wedge design. Sample size requirements for a parallel design are relatively modest; our example gave a minimum sample size of 20 clusters (ten per study arm) with each cluster providing 100 person-years of follow-up per year and a follow-up duration of 3 years. Although careful planning and substantial funding are required to run such a trial, the benefits of having a robust evidence-base from which to promote programmatic roll-out and/or further optimisation of the strategy should prove invaluable.

Supporting Information

Text S1 Statistical appendix containing: (1) a diagram of a parallel two-arm cluster randomised trial (PCRT) and a stepped wedge cluster randomised trial (SWCRT), (2) details regarding the determination of coefficients of variation for the Thailand data, and (3) details regarding the simulation study to compare PCRT versus SWCRT designs. (DOCX)

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Research article

Impact of Autocidal Gravid Ovitraps on Chikungunya Virus Incidence in *Aedes aegypti* (Diptera: Culicidae) in Areas With and Without Traps

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Abstract

Puerto Rico detected the first confirmed case of chikungunya virus (CHIKV) in May 2014 and the virus rapidly spread throughout the island. The invasion of CHIKV allowed us to observe *Aedes aegypti* (L.) densities, infection rates, and impact of vector control in urban areas using CDC autocidal gravid ovitraps (AGO traps) for mosquito control over several years. Because local mosquitoes can only get the virus from infectious residents, detecting the presence of virus in mosquitoes functions as a proxy for the presence of virus in people. We monitored the incidence of CHIKV in gravid females of *Ae. aegypti* in four neighborhoods—two with three AGO traps per home in most homes and two nearby neighborhoods without AGO mosquito control traps. Monitoring of mosquito density took place weekly using sentinel AGO traps from June to December 2014. In all, 1,334 pools of female *Ae. aegypti* (23,329 individuals) were processed by real-time reverse transcription PCR to identify CHIKV and DENV RNA. Density of *Ae. aegypti* females was 10.5 times lower (91%) in the two areas with AGO control traps during the study. Ten times (90.9%) more CHIKV-positive pools were identified in the nonintervention areas (50/55 pools) than in intervention areas (5/55). We found a significant linear relationship between the number of positive pools and both density of *Ae. aegypti* and vector index (average number of expected infected mosquitoes per trap per week). Temporal and spatial patterns of positive CHIKV pools suggested limited virus circulation in areas with AGO traps.

Key words: mosquito control, *Aedes aegypti*, arboviral transmission, invasive species, vector-borne pathogen

Although chikungunya (CHIKV), dengue (DENV-1, DENV-2, DENV-3, and DENV-4), yellow fever (YFV), and Zika (ZIKV) viruses originally circulate between nonhuman primates and forest mosquitoes in natural areas (Gubler 2002, Weaver and Reisen 2010), over time they have established independent transmission cycles between humans and domestic mosquitoes in urban areas (Musso and Gubler 2016). Currently, YFV outbreaks are mostly limited to areas where there is movement of the virus from enzootic foci to urbanized areas, whereas the sources of epidemics for the rest of these arboviruses primarily originate from other infected urban areas (Monath and Vasconcelos 2015). Lack of vaccines against CHIK, DENV, and ZIKV determines that vector control is the only available approach for the prevention and control of chikungunya, dengue, and Zika fevers.

Current approaches for the control of container *Aedes* (*Stegomyia*) mosquitoes involved in human-to-human transmission of these arboviruses, such as *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse), are elimination or modification of containers where mosquitoes develop, application of larvicides to containers, and spatial spraying of insecticides against adult mosquitoes. Residual insecticide applications are basically not being used against these vectors, with the exceptions of some limited use for focal control (around cases) in some countries (Ritchie et al. 2002). Widespread insecticide resistance against organophosphate and pyrethroid insecticides has been reported in *Ae. aegypti* and to a lesser extent in *Ae. albopictus* (Vontas et al. 2012). Interestingly, a variety of larvicides are effective against the immature stages of these mosquitoes, including biorational pesticides such as *Bacillus thuringiensis israelensis*, spinosad, juvenile hormone mimics, chitin synthesis inhibitors, and biodegradable oils (Barrera 2015).

The ongoing, unprecedented geographical expansion of CHIKV, DENV, and ZIKV (Bhatt et al. 2013, Weaver and Forrester 2015,

Higgs 2016) would indicate that vector control is not being effectively achieved or practiced. Current vector control approaches are based on a "seek and control" strategy to deliver vector control agents to places where it is thought that mosquitoes are located, such as by visiting houses to conduct source reduction, larviciding, and fumigation. The main limitations to that approach are relatively short-lived action of control measures (2–3 wk) necessitating reapplication of control measures at a frequency that most Vector Control Programs cannot afford, finding that a large fraction of houses cannot be treated because residents are not present or refuse treatment, the increasingly common finding that a large fraction of the mosquito population derives from cryptic aquatic habitats, and insecticide resistance (Barrera 2015).

Other approaches to vector suppression include luring mosquitoes to devices that result in passive or active killing (lure and control strategy) or by means of releasing modified mosquitoes making contact with local individuals of their own species to deliver a control agent (auto-dissemination strategy). Examples of this latter approach are the release of males carrying lethal genes, sterilized by irradiation, infected with *Wolbachia* bacteria or entomo-pathogenic fungi, or contaminated with pyriproxyfen (Scholte et al. 2004, O'Connor et al. 2012, Alphey et al. 2013, Bellini et al. 2013, Mains et al. 2015). The advantage of auto-dissemination approaches is that once the residents have given their consent, investing time and human resources asking for permission to enter and treat individual houses is unnecessary. Another promising approach not based on population suppression is to permanently replace a vector mosquito population with individuals that cannot transmit a particular arbovirus (Hoffmann et al. 2011, Aliota et al. 2016). These novel approaches are currently in field trials, but none have yet reached the stage of evaluating their impact on human disease.

Insect traps successfully suppress agricultural insect pest populations (Day and Sjogren 1994) and tsetse flies (Lindh et al. 2009), but their use as control tools against *Ae. aegypti* has been limited to some Vector Control Programs (Rapley et al. 2009). Several traps have been tested as control tools, including BG-Sentinel traps (Degener et al. 2014) and a variety of ovitraps targeting the eggs (Regis et al. 2013) or gravid females (Sithiprasasna et al. 2003, Kittayapong et al. 2008, Ritchie et al. 2008, Barrera et al. 2014a, Degener et al. 2015). Traps targeting ovipositing females eliminate those mosquitoes already fed on blood and possibly infected with arboviruses. A disadvantage of using traps as control tools in urban areas is the need to place traps in protected areas on private properties, thus requiring the consent and acceptance of individual residents. Another logistical factor is the need to deploy enough traps per residence in most of the houses to achieve area-wide population suppression (Degener et al. 2015). As with the other container-*Aedes* control tools, testing whether mosquito traps are useful for the prevention and control of arbovirus infections and human disease is necessary. Ideally, such studies use epidemiological and clinical data to evaluate the impact of the vector control measure, with adequate experimental methods like the cluster randomized design (Wolbers et al. 2012). However, such a study would require significant resources and conducting smaller studies, such as investigating the incidence of virus in mosquitoes in areas with and without control measures to gather preliminary evidence of efficacy that can reduce costs (Lambrechts et al. 2015).

This investigation used that approach to explore if the use of CDC autocidal gravid ovitraps for control (AGO traps; Mackay et al. 2013) could result in significant differences in the incidence of CHIKV in gravid females of *Ae. aegypti* between neighborhoods with and without traps. Local *Ae. aegypti* mosquitoes may get the virus from infectious residents or may be born with it (Agarwal et al. 2014) and a sudden increase and persistence of infected local mosquitoes is an indirect indicator of ongoing virus transmission among residents. AGO traps have been tested for their effectiveness at controlling populations of *Ae. aegypti* in two isolated neighborhoods in southern Puerto Rico; at one site since 2011 and at the other one since 2013 (Barrera et al. 2014a,b). The results of this ongoing, longitudinal entomological study have shown that the populations of *Ae. aegypti* are being kept 60–80% below expected levels, without presenting the frequent mosquito outbreaks observed in two nearby neighborhoods without control traps. After the first detection of CHIKV cases in Puerto Rico in May 2014 (Sharp et al. 2014), we used our weekly collections of mosquitoes to compare CHIKV virus incidence in *Ae. aegypti* in areas with and without AGO control traps to test the hypothesis that the presence of control traps limited local outbreaks of CHIKV. Given the observed significant reduction of virus incidence in mosquitoes in areas with traps, we propose values of *Ae. aegypti* density thresholds that reduced local CHIKV transmission in a nonimmune human population. A subsequent study of the prevalence of antibodies against CHIKV in residents of these communities showed significantly lower prevalence (50%) in areas with traps (Lorenzi et al. 2016).

Materials and Methods

The study took place from June to December 2014 in four neighborhoods in southern Puerto Rico. La Margarita (Intervention area I) was a relatively isolated community (17° 58′18″ N, 66° 18′10″ W; 3 m elevation) with 327 buildings (18 Ha) where three AGO control traps were deployed per home in 85–87% of homes (793 traps) since December 2011. Villodas was the other intervention area (Intervention area II) that was also relatively isolated from nearby communities (17° 58′13″ N, 66° 10′48″ W; 20 m elevation). Villodas had 241 houses (11 Ha), and we deployed three AGO control traps per home in 83–87% of homes (570 traps). Villodas served as a nonintervention area from December 2011 to February 2013 when control AGO traps were deployed as a partial cross-over intervention (Barrera et al. 2014b). Stationary sentinel AGO traps (SAGO) were uniformly distributed across La Margarita (44 traps) and Villodas (27 traps) to monitor local *Ae. aegypti* populations weekly.

As the study design did not require isolated control areas, the two nonintervention or reference areas, Arboleda and Playa, were part of larger neighborhoods. We deployed 30 SAGO traps in Arboleda (Nonintervention area I) in an area having 398 houses (17° 58′46″ N, 66° 17′23″ W; 10 m elevation; 21 Ha), whereas 28 SAGO traps were deployed in a sector of Playa (Reference area II) that had 269 houses (17° 57′59″ N, 66° 18′10″ W; 1 m elevation; 17 Ha). We serviced both control and sentinel AGO traps every two months and examined sentinel traps every week to collect and account for number of adult mosquitoes, species, and sex. Most of the *Ae. aegypti* females collected every week from SAGO traps in each of the four study sites were pooled (1–20 specimens per pool per site per week) and preserved at −80°C until they were processed by real-time reverse transcription-polymerase chain reaction (RT-PCR) to identify viral RNA of DENV and CHIKV.

Air temperature, relative humidity, and rainfall were recorded using meteorological stations (HOBO Data Loggers, Onset Computer Corporation, Boume, MA) located in the center of La Margarita, Villodas, and Arboleda. Because Playa and La Margarita were adjacent neighborhoods (200 m apart), we used the same meteorological data for both communities. We conducted the study

during the warmer and wetter season part of the year. Additional details of the study areas are available (Barrera et al. 2014a,b).

Detecting DENV and CHIKV in Mosquitoes by RT-PCR

Mosquito pools were homogenized using six 2.8-mm ceramic grinding beads (VWR, Radnor, PA) in a Qiagen TissueLyser (Qiagen, Germantown, MD) at 25 cycles per second for 5 min with 1% bovine serum albumin (pH 7.0), 1.5 ml of BA1 Diluent (2 mM L-glutamine, 1× M199-Hank's salts, 0.05 M Tris buffer; pH 7.5), 0.35 mg sodium bicarbonate, 100 units of penicillin, 100 µg of streptomycin, and 1 µg of Amphotericin B per ml. The homogenate was centrifuged (3 min at 8,000 rpm) and the supernatant removed and aliquoted into one tube for virus testing and another one for storage. RNA was extracted using a Qiagen M48 automated extractor and Qiagen MagAttract Virus Mini M48 kits. The presence of CHIKV and DENV ribonucleic acid (RNA) was detected by RT-PCR.

RT-PCR for chikungunya was adapted from Lanciotti et al. (2007), where each reaction contained 12.5 µl of 2× reaction mix, 6.35 µl nuclease free water, 0.25 µl of each primer (forward and reverse) at a concentration of 100 µM, 0.15 µl of a FAM labeled probe at a concentration of 25 µM, and 0.5 µl SuperScript III RT/Platinum Taq polymerase. Each reaction contained 5.0 µl of RNA template and amplified in 96-well plated on an Applied Biosystems 75000 Fast DX Real Time PCR Instrument. Dengue virus RNA was detected in multiplex using Invitrogen's Superscript III Platinum One-Step quantitative RT-PCR system (Santiago et al. 2013). Briefly, each DENV RT-PCR reaction contained 5.57 µl of nuclease free water, 12.5 µl 2× reaction mix, and 0.25 µl of SuperScript III RT/Platinum Taq polymerase. Primers were prepared at a solution of 100 µM, of which 0.25 µl of DENV type 1 and 3 primers, and 0.125 µl of DENV type 2 and 4 primers were added to the master mix. Thermocycling conditions consisted of three stages: 1) 30 min at 50°C, 2) 2 min at 95°C, and 3) 15 s at 95°C and 1 min at 60°C. Data were collected at the second step of stage 3, and samples with a Ct value of 37 or below were considered positive for the presence of virus.

Statistical Analyses

We investigated if the number of females of *Ae. aegypti* captured per trap per week was significantly different in areas with and without traps using a generalized linear mixed model analysis (GLMM). Rainfall (accumulated during the third and second week before each mosquito sampling), temperature (average of current and 2 wk before sampling), and relative humidity (average of current and 2 wk before sampling) were included as covariates. We used a negative binomial distribution model with log link and a first-order auto-regressive function for the covariance structure of the repeated measures. Study site and trap ID were included as random factors to account for trap variability. In addition, a generalized linear model (GLM) was employed to determine if the number of positive pools identified per site per week could be explained by the presence of AGO control traps (intervention vs. nonintervention sites) using the following covariates: average number of female *Ae. aegypti* per trap per week, rainfall, temperature, and relative humidity. The null hypothesis was that the number of positive pools detected every week was not statistically different in areas with and without control traps. The distribution probability function of the dependent variable was a Poisson with log link. Statistical analyses were performed using IBM SPSS Statistics 20 software (IBM Corporation, Armonk, NY). Maximum likelihood minimum infection rates of mosquitoes and two sample tests were calculated using PooledInfRate version 4.0 (Biggerstaff 2016). The Vector Index (VI), an indicator of the expected number of infected mosquitoes per trap per week, was calculated as the proportion of infected mosquitoes times the average number of mosquitoes captured per trap per week (Jones et al. 2011).

Results

Mosquito Dynamics

In total, 26,251 females and 3,649 males of *Ae. aegypti* were captured from 3,859 traps × weeks between June 11 and December 31, 2014 in the four study areas. Most *Ae. aegypti* females (55.2%) and males (68%) were captured in Playa, followed by Arboleda (33.5% females, 24.3% males), La Margarita (7.5%, 4.8%), and Villodas (3.8%, 2.9%; Table 1). *Aedes mediovittatus* (Coquillett) was captured in AGO traps but at very low densities (Table 1). *Culex quinquefasciatus* Say was also commonly captured, but their numbers were not recorded in this study. The GLMM analysis comparing number of *Ae. aegypti* per trap per week was significant ($F_{4,3814} = 142.6$, $P < 0.001$), with significant effects of the presence of AGO control traps ($t = 20.4$, $P < 0.001$), accumulated rainfall ($t = 11.4$, $P < 0.001$), and temperature ($t = -4.8$, $P < 0.01$). Means estimated by the model were 11.6 and 1.1 females of *Ae. aegypti* per trap per week in nonintervention and intervention areas, respectively (fixed predictors: rainfall = 30.4 mm; temperature = 27.7°C; relative humidity = 75.7%). Thus, as an average, there were 10.5 times more *Ae. aegypti* females (91%) in areas without AGO control traps. The coefficient for accumulated rainfall indicated an average increase in the number of female *Ae. aegypti* per trap per week of one specimen per mm of rainfall. It can be observed that the numbers of adult females increased following corresponding increases in rainfall, particularly in the study sites without control traps (Arboleda, Playa; Fig. 1).

Table 1. Number of *Ae. aegypti* and *Ae. mediovittatus* mosquitoes captured in SAGO traps and metrological variables (mean ± SE) of the study sites registered from June to December 2014 in southern Puerto Rico

Study site	*Ae. aegypti* females	*Ae. aegypti* males	*Ae. mediovittatus* females	*Ae. mediovittatus* males	Avg temp (3 previous weeks; °C)	Accumulated rainfall (2nd and 3rd weeks before sampling; mm)	Average relative humidity (3 previous weeks; %)
La Margarita	1,963	173	13	1	28.0 ± 0.2[a]	30 ± 6[a]	75.3 ± 0.5[a]
Villodas	993	102	1	0	27.6 ± 0.2	41 ± 8	77.1 ± 0.6
Arboleda	8,807	887	3	0	27.1 ± 0.2	20 ± 4	75.4 ± 0.5
Playa	14,488	2,487	9	0	28.0 ± 0.2[a]	30 ± 6[a]	75.3 ± 0.5[a]
Total	26,251	3,649	26	1	27.7 ± 0.1	30 ± 3	75.8 ± 0.3

[a]Data came from the same meteorological station because these communities are adjacent.

Detection of DENV and CHIKV in *Ae. aegypti*

A total of 1,334 pools of *Ae. aegypti* females were collected and analyzed by RT-PCR to identify DENV and CHIKV RNA in the four study sites. Mosquito pools could not be collected in 4 wk out of the 30 wk of the study because of a shortage of personnel (July 1 and 7, August 5 and 26). None of the pools were positive for DENV. In total, 55 pools tested positive for CHIKV, for an overall infection rate of 2.41 mosquitoes per thousand (1.83–3.11 95% CI; Table 2). The first positive pool was registered on August 19 and the last on December 24. Using data only from August to December, the overall infection rate would be 3.24 (2.46–4.18; 905 pools, 17,500 specimens). The resulting number of pools and mosquitoes processed varied per site according to their local abundance (Table 2). Ten times more CHIKV-positive pools were identified in the nonintervention areas (50/55 pools or 91%; Playa, Arboleda) than in intervention areas (5/55 or 9%; La Margarita and Villodas; Table 2). The CHIKV minimum infection rates in each of the four sites were similar, with overlapping confidence intervals (Table 2). A two-sample test of the difference in infection rates between sites with the lowest (1.75) and highest (2.40) infection rates was not significant ($D = -0.75$; -2.47–2.32; $P > 0.05$). None of the 16 pools of *Ae. mediovittatus* was positive for DENV or CHIKV.

The results of the GLM analysis comparing number of positive pools per site per week between intervention and nonintervention areas were significant (Wald's $\chi^2 = 24.1$, df $= 1$, $P < 0.01$). Positive pools were detected for seven consecutive weeks out of 10 weeks showing positive pools in Playa (September 23–November 4), five out of 10 weeks in Arboleda (October 7–November 4), two out of three weeks in La Margarita (October 21–29), and no consecutive positive pools were detected in Villodas (Fig. 1). Consecutive virus detections in nonintervention areas disappeared when mosquito densities decreased to 2.4–4.4 female *Ae. aegypti* per trap per week on November 12, 2014 (Figs. 1 and 2). The density of *Ae. aegypti* in intervention areas were at or below three females per trap per week (Fig. 2). Most positive pools were registered between October 8–22, and they were scattered across the entire Playa and Arboleda communities (Figs. 3 and 4). Maps of the locations of positive pools in the intervention communities were not possible to draw, because given their lower mosquito densities, pools had to be made from specimens collected in many or most of the traps. For example, the total number of female *Ae. aegypti* mosquitoes collected in Villodas on the week of November 4 in all 27 sentinel AGO traps was 19, so that just one pool was made which was positive for CHIKV.

Rainfall peaked twice during the study; the first and larger peak during August–September associated with corresponding increases in *Ae. aegypti* captures and CHIKV detections, and a second peak in November associated with increases in mosquito densities but scattered virus detections (Fig. 1). The densities of *Ae. aegypti* in nonintervention areas were well above those observed in intervention areas most of the time (Fig. 2). In spite of the limited rainfall recorded in June and July, the density of *Ae. aegypti* in nonintervention areas stayed at relatively high levels (8.5–14.2). The overall VI, or expected number of infected mosquitoes per trap per week, in each of the two intervention sites (0.003) was eight and 14 times smaller than in the noninterventions sites Arboleda (0.024) and Playa (0.043), respectively (Table 2). The number of positive pools and average density of *Ae. aegypti* females per trap per week

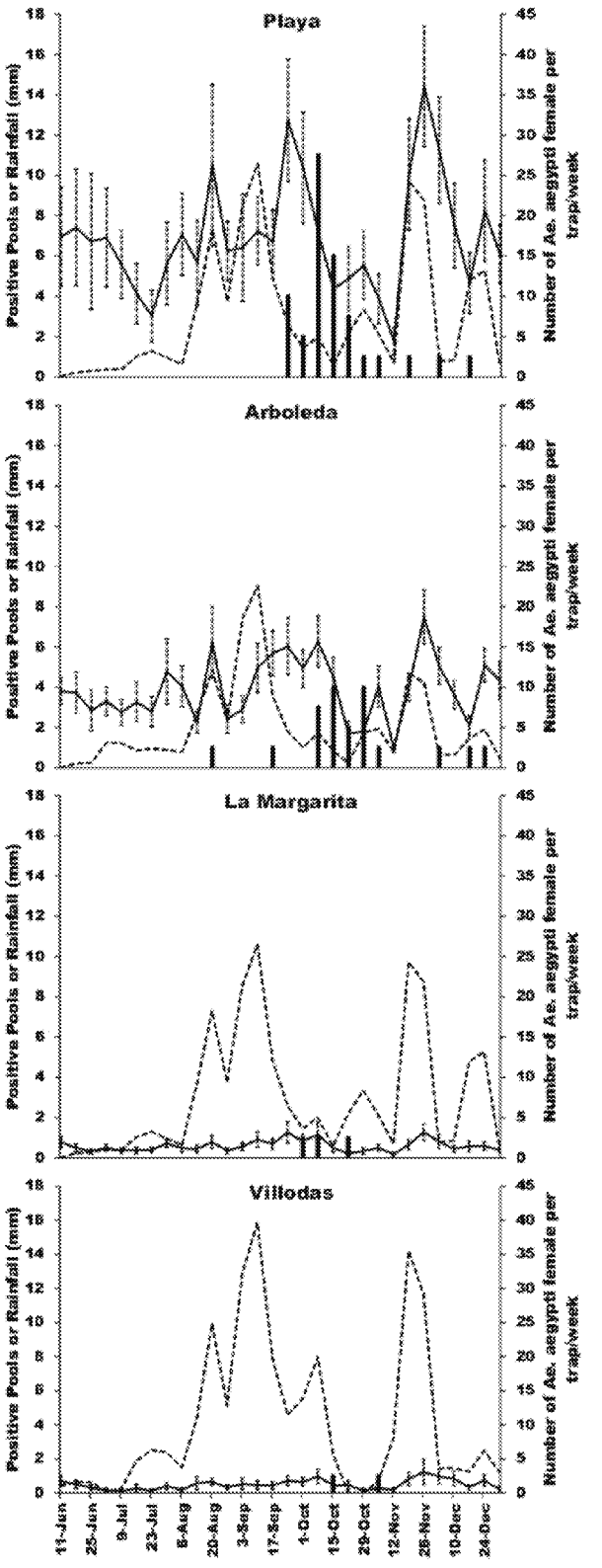

Fig. 1. Average number of female *Ae. aegypti* per sentinel AGO trap, CHIKV-positive mosquito pools, and accumulated rainfall (third and second week before sampling) per week in nonintervention (Playa, Arboleda) and intervention areas (La Margarita, Villodas) during the second half of 2014 in Puerto Rico.

Table 2. Number of CHIKV-positive pools of female *Ae. aegypti*, infection rates (per thousand mosquitoes), average pool size, number of tested mosquitoes, and average females trapped per week in SAGO traps in each of the study sites in southern Puerto Rico, from June to December 2014

Study site/Treatment	CHIKV-positive pools	CHIKV infection rate (× 1000; 95% CI)	Pools (avg mosquitoes/pool)	*Ae. aegypti* females in pools	Average (± SE) density of female *Ae. aegypti* per trap per week	VI (avg infected mosquitoes/ trap/week)
La Margarita/Intervention	3	1.75 (0.46–4.72)	104 (17)	1,730	1.5 ± 0.1	0.003
Villodas/Intervention	2	2.13 (0.38–6.98)	65 (15)	950	1.2 ± 0.1	0.003
Arboleda/Nonintervention	19	2.46 (1.53–3.77)	443 (18)	7,881	9.8 ± 0.2	0.024
Playa/Nonintervention	31	2.48 (1.72–3.48)	722 (18)	12,768	17.4 ± 0.6	0.043
Total	55	2.41 (1.83–3.11)	1,334	23,329	6.8 ± 1.0	0.016

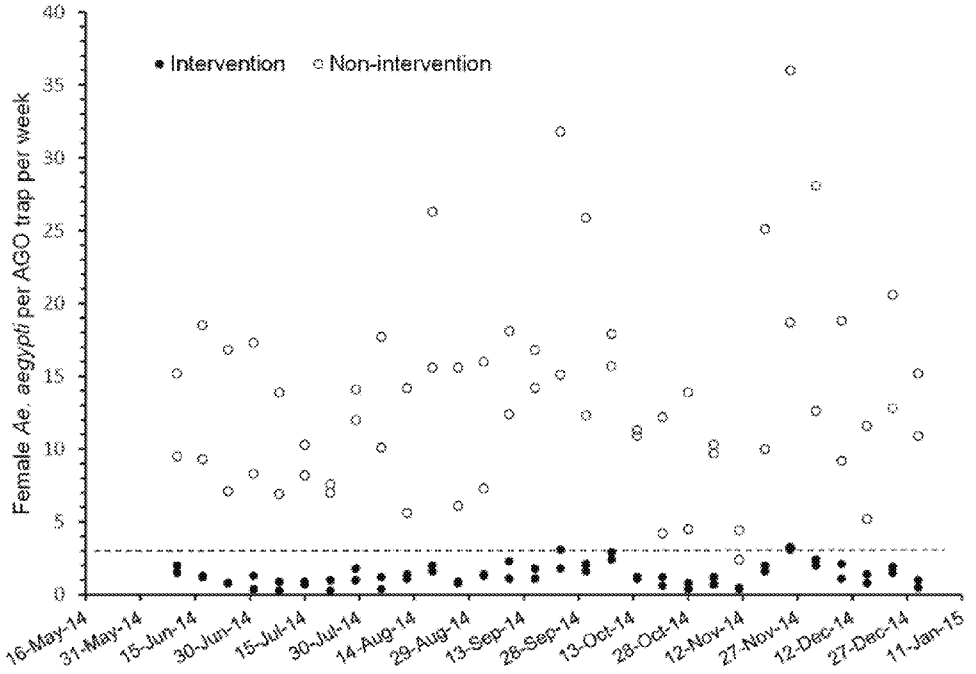

Fig. 2. Average number of female *Ae. aegypti* per trap per week in intervention (solid dots) and nonintervention (open dots) areas in Puerto Rico from June to December 2014. The dotted line drawn at three females per trap per week separates most average captures between intervention and noninterventions areas.

in the study locations had a highly significant linear relationship (Fig. 5; $r = 0.998$; $P < 0.01$). The correlation between number of positive pools and VI was similar ($r = 0.998$; $P < 0.01$).

Discussion

We have shown that using three CDC AGO traps per home in most homes (>85%) per neighborhood caused sustained reductions of *Ae. aegypti* captures in the order of 60–80% for several years (Barrera et al. 2014a,b). The purpose of the current investigation was to determine if mosquito population reduction in areas with traps was sufficient to prevent or limit the extent of local outbreaks of CHIKV. The hypothesis was that AGO control traps reduce the incidence of CHIKV in mosquitoes as a result of lowered vector densities. *Aedes aegypti* mosquitoes can only acquire the virus from infected persons, and thus virus detection in *Ae. aegypti* reflects infections in people living nearby. This approach to monitoring virus circulation is not common because the proportion of mosquitoes infected with arboviruses is generally very low, thus requiring large samples, and *Ae. aegypti* is typically a low-abundance mosquito (Barrera 2015). An advantage of using gravid traps to monitor infected mosquitoes is that their infection rate should be higher than in samples of adult mosquitoes captured using traps for host-seeking mosquitoes, which include nulliparous noninfectious females.

The results show that the densities of female *Ae. aegypti* were about 10 times lower in neighborhoods with control traps than in two nearby neighborhoods without control traps. Also, increases in mosquito density following rains were limited in intervention sites in comparison with the large increases observed in nonintervention neighborhoods. The total number of CHIKV positive mosquito pools detected was also about 10 times larger in nonintervention neighborhoods, showing a significant linear relationship with the density of female *Ae. aegypti* per trap per week among study sites. Interestingly, the minimum infection rate was similar across study sites. For this reason, the VI, or expected number of infected mosquitoes, showed the same linear relationship with the total number

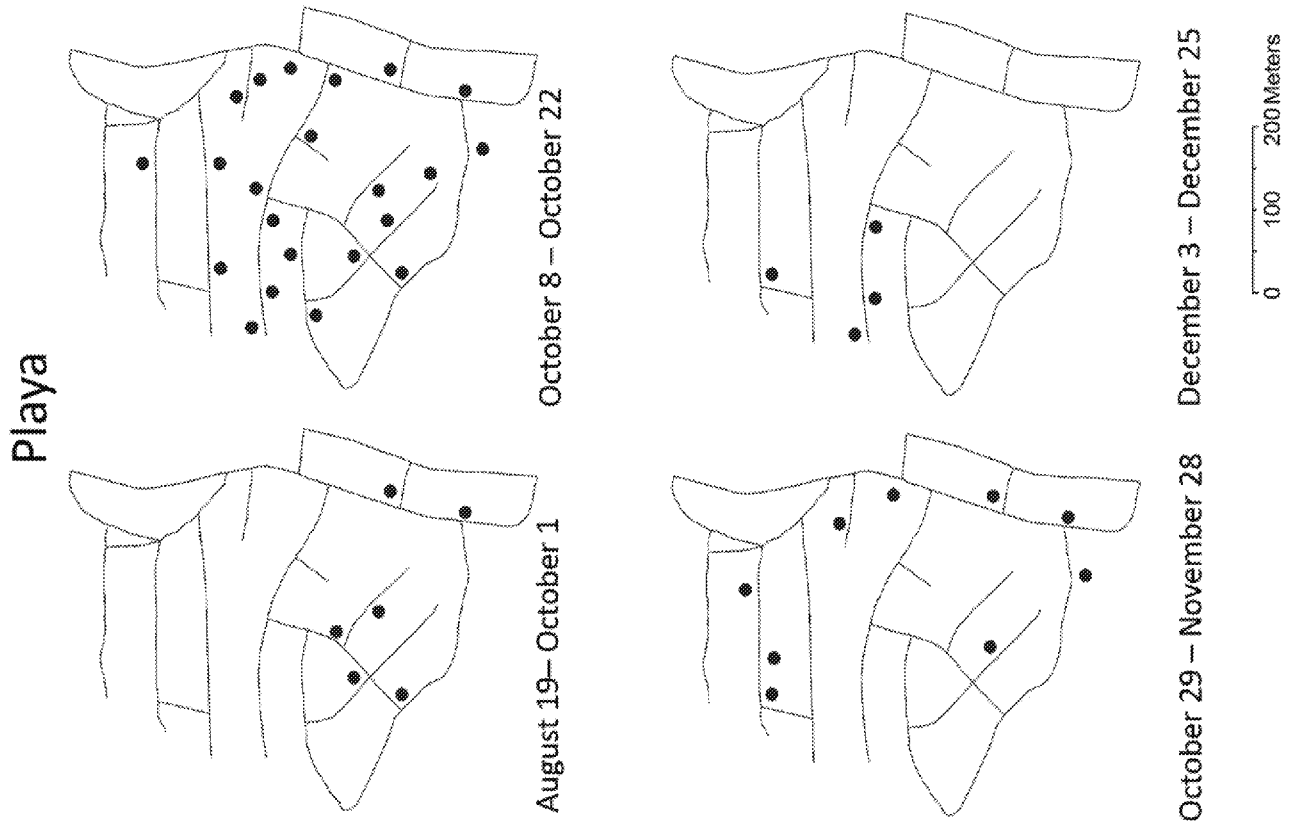

Fig. 3. Map of Playa community showing streets and locations of traps with CHIKV-positive mosquito pools from June to December 2014 in southern Puerto Rico.

Fig. 4. Map of Arboleda community showing streets and locations of traps with CHIKV-positive mosquito pools from June to December 2014 in southern Puerto Rico.

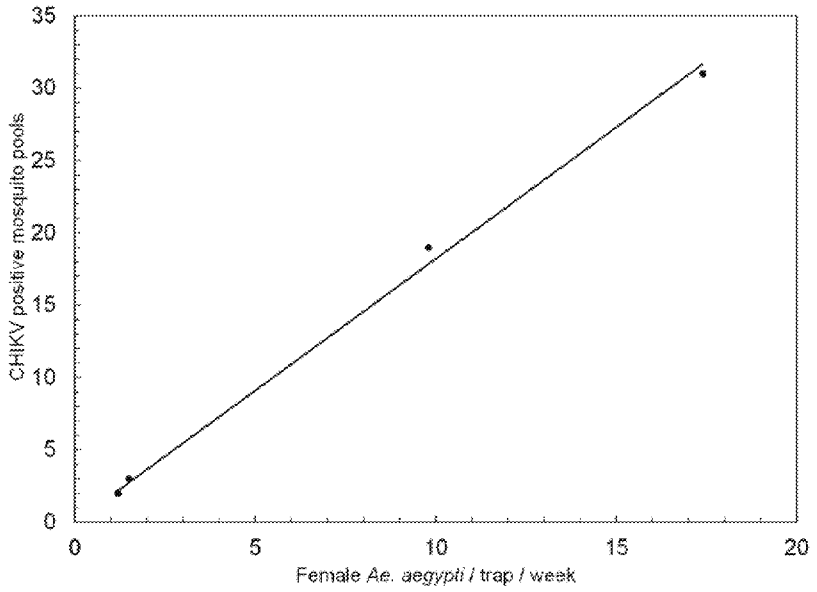

Fig. 5. Relationship between number of CHIKV-positive mosquito pools and average number of female *Ae. aegypti* per trap per week in all study areas from June to December 2014.

of positive pools as mosquito density. Perhaps, the most important observation was the presence of CHIKV-positive pools in consecutive weeks in nonintervention areas, interpreted as evidence of sustained local transmission. By contrast, positive pools in intervention areas were very few and scattered, interpreted as lack of sustained transmission or outbreak. Results obtained from a subsequent cross-sectional investigation of the prevalence of IgG CHIKV antibodies in residents of the study sites showed significantly lower prevalence in areas with AGO traps (Lorenzi et al. 2016). These results confirm that studies of the incidence of arboviruses in mosquitoes can be a proxy for human infections. The infection rates observed in this study (1.8–4.2 per thousand) were lower than those observed using AGO traps around confirmed CHIKV cases (8 per thousand) in various neighborhoods in Puerto Rico during 2014 (G.E.F., R.R.H, R.B., unpublished data). Infection rates found in this study were lower than in other reports, but comparisons are difficult to establish because of the use of different capture and sampling techniques (Sang et al. 2008, Diaz-Gonzalez et al. 2015, Dzul-Manzanilla et al. 2015).

Proving the efficacy of vector control interventions against arboviruses in natural settings is challenging due to the short duration of infections in humans and the transient occurrence of local outbreaks, which result from buildup of life-long immunity and exhaustion of susceptible hosts. This limitation is particularly important in small groups of people, such as those one would use in cluster randomized designs. For these reasons, alternative and more affordable approaches have been suggested as a way to test the effectiveness of vector control tools (Lambrechts et al. 2015). The approach followed in this investigation mirrors an observational cohort study, with one group having a presumed protective intervention and the outcome of exposure to circulating viruses followed over time in mosquito populations rather than people. We anticipated exposure to DENV and CHIKV because dengue viruses have been endemic in Puerto Rico since 1980s (Barrera 2010) and the first ever detection of local transmission of CHIKV was in May 2014 (Sharp et al. 2014). The current spread of Zika virus in Puerto Rico and the Americas suggest its eventual occurrence in the same urban areas affected by DENV and CHIKV in the past. This methodology is applicable then to testing the effectiveness of vector control measures against all three viruses that share the same transmission cycle.

The results from this investigation suggest that local transmission of CHIKV in the two communities was more likely when the density of *Ae. aegypti* was larger than three females per trap per week. Even in the nonintervention areas, the presence of virus in gravid mosquitoes decreased and became more sporadic when the density fell to 2.4–4.4 females of *Ae. aegypti* per trap per week in November 2014. Mosquito densities around confirmed CHIKV cases in three neighborhoods with positive pools of female *Ae. aegypti* in Puerto Rico in 2014 were 5.3–20.5 per AGO trap per week and 4.3 in the neighborhood where no positive pools were found (CDC, unpublished data). Ritchie et al. (2004) observed that no DENV was observed in mosquitoes and human cases dropped, when the density of gravid *Ae. aegypti* females fell below 0.5 females per sticky trap per week. Because their trap was smaller than the AGO trap, fewer mosquitoes are expected to signal a possible threshold for transmission. In a previous study, we compared captures in BG-Sentinel and AGO traps in the four study areas, showing a significant, positive nonlinear relationship (Barrera et al. 2014a). The equivalent density in BG-Sentinel traps to three females per AGO trap per week is one female per trap per day. We are not aware of any previous studies using BG-Sentinel traps where a threshold for arbovirus transmission has been proposed. Active DENV transmission was reported during the dry and cooler season in San Juan city, when the density of *Ae. aegypti* in BG-Sentinel traps was the lowest but still between 2–3 females per trap per day (Barrera et al. 2011). Additionally, a comparison of captures in AGO traps and paired ovitraps in Puerto Rico was significant, with a positive nonlinear relationship (Mackay et al. 2013). The calculated equivalent egg density in paired ovitraps to three females per AGO trap per week is six eggs per day. Mogi et al. (1990) reported the appearance of dengue hemorrhagic fever cases when the density of eggs of *Ae. aegypti* was larger than three eggs per ovitrap per day, which is similar to our figure of six eggs per pair of ovitraps. Investigators have suggested *Ae. aegypti* density thresholds for arbovirus transmission using larval indices (Connor and Monroe 1923, Brown 1974). Modeling shows that threshold densities vary with temperature, frequency, and amount of virus

importation, and herd immunity (Focks et al. 2000). Generally, higher thresholds or more mosquitoes would be required to cause an outbreak at lower temperature, lower frequency of virus importation, and higher levels of herd immunity. Other factors may also come into play affecting threshold densities by reducing vectorial capacity (Newton and Reiter 1992), such as smaller contact rates between mosquitoes and people with the use of screens and other personal protection measures (Waterman et al. 1985). The ability to define minimum numbers of *Ae. aegypti* females protective against rampant arboviral outbreaks is important, so vector control programs can have clearly defined goals, but defining such thresholds requires additional research. The relatively recent availability of practical tools for monitoring the adult *Ae. aegypti* population will facilitate such a task (Barrera 2016).

This investigation used AGO traps for surveillance and control purposes, thus in treatment areas, sentinel traps were surrounded by many control traps. A concern is that mosquito density in surveillance traps may provide an underestimation of the real mosquito density in the presence of control traps, which would then reflect an overestimated reduction in vector density. We addressed that concern in an earlier work (Barrera et al. 2014a), where we compared weekly captures of female *Ae. aegypti* in sentinel AGO and in modified BG traps (Barrera et al. 2013) in areas with and without control traps for over one year. The results showed significant, nonlinear positive relationships between captures in both traps, which were similar in areas with and without AGO control traps. For that reason, we are confident that AGO traps are reliable surveillance tools in the presence of control traps.

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PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Male Mosquitoes as Vehicles for Insecticide

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Abstract

Background

The auto-dissemination approach has been shown effective at treating cryptic refugia that remain unaffected by existing mosquito control methods. This approach relies on adult mosquito behavior to spread larvicide to breeding sites at levels that are lethal to immature mosquitoes. Prior studies demonstrate that 'dissemination stations,' deployed in mosquito-infested areas, can contaminate adult mosquitoes, which subsequently deliver the larvicide to breeding sites. In some situations, however, preventative measures are needed, e.g., to mitigate seasonal population increases. Here we examine a novel approach that combines elements of autocidal and auto-dissemination strategies by releasing artificially reared, male mosquitoes that are contaminated with an insecticide.

Methodology

Laboratory and field experiments examine for model-predicted impacts of pyriproxyfen (PPF) directly applied to adult male *Aedes albopictus*, including (1) the ability of PPF-treated males to cross-contaminate females and to (2) deliver PPF to breeding sites.

Principal Findings

Similar survivorship was observed in comparisons of PPF-treated and untreated males. Males contaminated both female adults and oviposition containers in field cage tests, at levels that eliminated immature survivorship. Field trials demonstrate an ability of PPF-treated males to transmit lethal doses to introduced oviposition containers, both in the presence and absence of indigenous females. A decline in the *Ae. albopictus* population was observed following the introduction of PPF-treated males, which was not observed in two untreated field sites.

Conclusions/Significance

The results demonstrate that, in cage and open field trials, adult male *Ae. albopictus* can tolerate PPF and contaminate, either directly or indirectly, adult females and immature breeding sites. The results support additional development of the proposed approach, in which male mosquitoes act as vehicles for insecticide delivery, including exploration of the approach with additional medically important mosquito species. The novelty and importance of this approach is an ability to safely achieve auto-dissemination at levels of intensity that may not be possible with an auto-dissemination approach that is based on indigenous

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females. Specifically, artificially-reared males can be released and sustained at any density required, so that the potential for impact is limited only by the practical logistics of mosquito rearing and release, rather than natural population densities and the self-limiting impact of an intervention upon them.

Author Summary

Approximately half of the human population is at risk of dengue. Additional mosquito borne pathogens, *e.g.*, chikungunya, are spreading globally, as are important mosquito vectors. In the absence of approved vaccines, therapeutant or prophylaxis, vector control remains the only means to combat multiple mosquito-borne pathogens. Auto-dissemination strategies have attracted attention as a method to reduce mosquito populations and benefit from mosquito behavior, in which a female mosquito visits multiple breeding sites. As practiced currently, 'dissemination stations' are attractive to adult females, which enter the station, become contaminated with a juvenile hormone analogue (JHA), exit and then contaminate breeding sites with levels of JHA that are lethal to immature mosquitoes. The auto-dissemination method is particularly attractive for those species that breed within small, cryptic sites, which serve as refugia from existing insecticidal measures. Here we examine mathematically and empirically, a novel approach that is not station-based, but which integrates elements of autocidal control. Specifically, the approach would release JHA-contaminated adult male mosquitoes, which do not bite or transmit pathogens. The males deliver JHA to breeding sites, either directly or indirectly, *i.e.*, via the cross-contamination of females, which subsequently transfer JHA to breeding sites. The examined autocidal method can be used preemptively, *e.g.*, in areas with low densities of indigenous mosquitoes and in advance of the natural population increase. Unlike auto-dissemination approaches that rely upon the indigenous population, an approach based on artificially-reared males can made more intensive, because the number of males released is limited only by the logistics of male rearing and release and methods for mass-production of mosquitoes are developed already.

Introduction

Mosquito control remains the only means available to combat some medically important, vector-borne pathogens, such as West Nile, Dengue and Chikungunya viruses, because no approved vaccine, therapeutant or prophylaxis exist [1,2]. Chemical insecticides are used most commonly in mosquito control, with formulations that include larvicides and adulticides (*e.g.*, space sprays, residual indoor applications, and insecticide-treated bed nets) [3]. With each of these approaches, however, their efficacy can be reduced by an inability to achieve adequate coverage that is needed to effectively reduce pathogen transmission [4-6].

Larvicides are applied to aquatic habitats of developing immature mosquitoes ('breeding sites') and are demonstrated to reduce mosquito-borne disease transmission [7-9]. However, in some contexts, its implementation at a programmatic level can be difficult [10]. Specifically, aquatic habitats include a variety of types, many of which can be small, sheltered and difficult to locate and treat ('cryptic breeding sites') [11]. Because financial and human resources are limited, it can be difficult to achieve a coverage level sufficient to reduce disease [5,12]. Area-wide broadcasting of larvicides can be used to improve coverage, via aircraft and vehicle-

mounted sprayers, but in some situations, broadcasting is constrained by environmental regulations or restrictions, community concerns and mosquito resistance to the active ingredients of existing larvicides [13-16]. Furthermore, some larvicide formulations are expensive, and the large quantities required for broadcasting strategies can be cost prohibitive.

Auto-dissemination has attracted attention due to its potential to address important gaps with existing mosquito control methods. Auto-dissemination is a method of pesticide 'self-delivery,' which is premised upon the use of insects as the delivery agent. Insects carrying small amounts of insecticide can deliver an active ingredient to cryptic refugia, rather than human applicators, and this method can require less pesticide relative to broadcasting. For this reason, auto-dissemination approaches have become an important pesticidal method for termites, beetles, and moths [17].

Auto-dissemination approaches are being explored for mosquitoes [18,19]. Proposed auto-dissemination methods are based on the behavior of adult mosquitoes and their attraction to breeding sites, including cryptic sites that human operators may often fail to find. As currently practiced, auto-dissemination consists of placing artificial adult resting sites ('dissemination stations') that are (1) attractive to adult mosquitoes and (2) are treated with a persistent juvenile hormone analogue (JHA) [20]. Upon entering the dissemination station (Fig. 1A), the adult mosquitoes become contaminated with the JHA, which is not acutely toxic to the adult. The JHA is lethal to immature mosquitoes, when their breeding sites become contaminated by the females that arrive to lay eggs and introduce the JHA. An additional approach is based on treated bed nets [21].

Models predict a multiplicative ability of the auto-dissemination approach to achieve high breeding site coverage, despite covering a relatively small proportion of the resting sites. The coverage of breeding sites (C_h) is related to the coverage of adult resting sites (C_r), the duration for which habitats remain unproductive after contamination (U), the number of ovipositions by the mosquito population (O) relative to the number of habitats (H), and the mean number

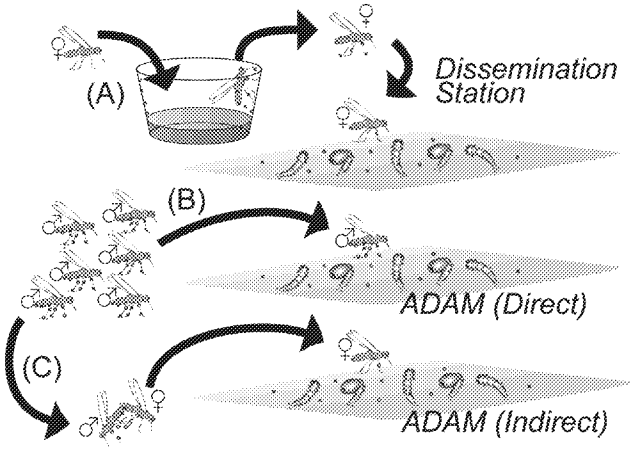

Figure 1. Diagram comparing the auto-dissemination station-based approach with the ADAM approach. In (A), an auto-dissemination station is attractive to indigenous female mosquitoes (grey), which enter and become contaminated with a persistent juvenile hormone analogue (PPF) that does not harm the adult. The PPF-contaminated females (black) exit the trap and transfer the PPF to immature mosquito breeding sites. In (B and C), the ADAM approach is based on manufacturing adult male mosquitoes that are dusted with PPF (black), which are released into a treatment area. The PPF-treated males can then (B) directly transfer PPF to immature mosquito breeding sites and (C) indirectly transfer PPF by cross-contaminating indigenous females, which subsequently transfer the PPF to breeding sites.

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of contaminated ovipositions required to render a breeding site unproductive (Ω) [19,22].

$$C_h = 1 - e^{-C_r UO/H\Omega} \quad (1)$$

This relationship shows that a majority of breeding sites can be affected, even when treating a minority of resting sites, if the pesticide is durable ($U \geq 7$ days), the mosquito abundance and habitat availability is such that breeding sites are contaminated more than once per day ($O/H \geq 2$) and one contamination is sufficient to render a habitat unproductive ($\Omega = 1$).

In addition to modeling, field trials by multiple research groups have demonstrated the efficiency of the auto-dissemination approach, showing that (1) mosquitoes become contaminated using different dissemination station designs and (2) that the contaminated mosquitoes can transmit lethal doses of the pesticide [19,20]. Importantly, prior studies demonstrate also that (3) adult male mosquitoes are attracted to and are contaminated by auto-dissemination stations, (4) that males can venereally transfer JHA to females upon mating and (5) that the venereally-contaminated females can subsequently transfer lethal concentrations of JHA to breeding sites [18].

The most commonly used JHA in the auto-dissemination approach is pyriproxyfen (PPF), which does not affect contaminated adults, *i.e.*, it is neither lethal nor repellant [23]. In contrast to adults, the concentration required to prevent mosquito development (LC_{50}) is 0.012 parts per billion [23]. At this rate, 32mg of PPF would be adequate to treat an olympic-size swimming pool (2.5 million liters). And 1/1,000th of the dry weight of a mosquito adult would be adequate to treat a 200ml breeding site [24]. The residual activity of PPF is four months in water [25]. Little resistance to this chemical class has been observed in mosquitoes [26,27]. PPF is relatively safe for non-targeted organisms, including vertebrates [28]. The World Health Organization has approved PPF for use in drinking water [29].

Here, we use models to consider the limitations of current station-based auto-dissemination approaches and to propose an additional auto-dissemination method that is based upon the release of PPF-treated male mosquitoes (Fig. 1). Inundative male releases are feasible, because in mosquitoes, the males do not bite or transmit pathogens to the human population [30]. The approach of releasing PPF-treated male mosquitoes is subsequently referred to as "Auto-Dissemination Augmented by Males" (ADAM). We examine empirically (1) the effects of PPF on the survival of male *Aedes albopictus* which serve to contaminate breeding sites, (2) the ability of treated males to directly contaminate larval breeding sites, *i.e.*, even without females and (3) the ability of treated males to transfer PPF to females, at dosages adequate to lethally contaminate larval breeding sites. The results encourage additional examination and development of this approach as an additional tool against important mosquitoes.

Methods

Ae. albopictus mosquitoes used in experiments were from a colony established in Lexington, Kentucky in 2011 and named the "Wildcat" (WC) strain. Larvae were fed with a 60g/L liver powder (ICN Biomedicals, CA, USA) suspension *ad libitum*. Adults were held in 24.5 cm^3 cages (MegaView Science Co., Taichung, Taiwan) and provided a constant supply of 10% sucrose. Adult mosquitoes used in experiments were between one and two days post emergence. The PPF treatment consisted of a 30:70% mixture of Esteem 35 WP IGR (Valent Biosciences, IL, USA) and DayGlo (Dayglo Color Corp., Cleveland, OH), respectively. The PPF was applied to mosquitoes housed in a 1 L enclosed cardboard container using a PowerPuff insufflator for approximately 5 sec (Gremar Power Puff 898, Gremar Inc., W. Des Moines, IA). Dayglo is routinely used for mosquito marking (*e.g.*, mark release recapture experiments) [31,32] and facilitates the subsequent tracking of the dust mix. We note that PPF is a pupacide. However,

PPF is registered and commonly referred to as a 'larvicide,' and therefore we use the latter terminology here.

For longevity studies, adult male mosquitoes were dusted with PPF (treatment) or left undusted (control). Three replicate control and treatment groups of 15 males/each were put into cages with a 10% sucrose solution and monitored for adult survivorship.

For field cage trials, ten oviposition cups were placed within each field cage (10'x12'; Ozark Trail, CA, USA), along with a 10% sucrose solution. Five oviposition cups/cage were covered with bridal veil to exclude mosquito entry. The remaining ovisites were identical, but uncovered. Ovisites consisted of black 0.5 L plastic cups (Solo Cup Co., USA) lined with germination paper (Anchor Paper Co., USA) and with 250 ml water.

For the field cage experiments, young adult male mosquitoes ($\leq$2d post eclosion) were treated with the Esteem/DayGlo mixture, as in the longevity study, and then introduced into cages (50 males/cage). Twenty-four hours after male release, 50 newly-eclosed virgin females were added to the field cages. Five days after establishment, all adults were evacuated from the field cages using a modified aspirator [33]. Adults were killed by freezing and observed for dust. Oviposition cups and associated egg papers also were examined for dust.

To test for the presence of larvicidal activity, bioassays were performed in 20 ml scintillation vials (#986540; Wheaton Millville, NJ) with 15 ml water, 0.2 ml liver powder solution and four second instar WC larvae. For bioassays of the adults that were removed from field cages, the removed adults were killed by freezing and then placed individually into bioassay vials, and assays were observed for immature survivorship. For bioassays of ovisites, each cup was separated into three components: water, germination paper lining and cup. For bioassays of ovisite water, two replicate 15 ml water samples were drawn from each ovisite and combined with 0.2 ml liver powder solution and four second instar WC larvae in scintillation vials, and immature survivorship was monitored. To examine for PPF on germination papers, each paper was removed from the ovisite and then submerged in 200 ml water with four second instar WC larvae and 1 ml liver powder solution, and immature survival was monitored. With the water and germination papers removed, each ovisite was examined for PPF by rinsing with 200 ml water, and 15 ml of the resulting rinsate was combined with 0.2 ml liver powder solution and four second instar WC larvae in scintillation vials, and immature survivorship was monitored.

Negative control assays consisted of an undusted male adult added to 15 ml water, 0.2 ml liver powder solution and four second instar WC larvae in a scintillation vial. Positive control assays were the same as negative control assays, but with the addition of a single male, freshly dusted with PPF powder.

Field studies were conducted at two sites in Lexington, KY. The adult population was monitored via weekly 24 hour collections using BG traps (Biogent Sentinel, Regensburg, Germany). Artificial ovisites were as described for field cages. Water samples were removed weekly from ovisites and bioassayed as described above. In the second field study, 10-minute landing collections were conducted to observe for *Ae. albopictus* females. WC males introduced at field sites were treated with the Esteem/DayGlo mixture, as described in the longevity study.

Statistical analyses were performed using JMP 9.0.1 and SAS 5.1 software (SAS Institute, Cary, NC). Kaplan-Meier, Log-Rank was used for analysis of adult longevity. Non-parametric analysis was used (Kruskal-Wallis and Wilcoxon) to examine results of bioassays. To assess population trends following treatment, a repeated measures ANOVA was conducted, followed by a linear regression of the female adult number [LN(females+1)] by Collection Week. To examine for an effect of proximity to release site, a linear regression was made between bioassay lethality and distance from the release point.

Results

Mathematical Consideration

As currently practiced against mosquito populations, models predict that station-based auto-dissemination relies upon a vigorous, naturally-occurring mosquito population. Specifically, indigenous mosquitoes must enter the station, become contaminated with the larvicide and then deliver the larvicide to breeding sites. Mosquitoes are the vehicle for the larvicide; therefore, in areas with lower mosquito densities, the larvicide may not be effectively delivered to breeding sites. Using the model above (Equation 1), the efficacy against the mosquito population is directly related to the number of ovipositions per population (O) [34]. Therefore, the model predicts that the impact of this approach to affect potential breeding sites will be limited, until there are adequate adults to become contaminated and transmit the pesticide (S1 Fig.). If these predictions are correct, this can limit station-based approaches as a preventative control tool. Prior laboratory work examining the relationship between adult mosquito number and accumulation of pesticide support model predictions [19].

The model predicts also that a station-based approach based solely upon naturally-occurring mosquitoes can be a victim of its own success. This is evident from the endogeneity within Equation 1, in which the coverage term on the left side of the equation is interdependent with the mosquito density term on the right side of the equation. Specifically, when introduced into areas of high mosquito activity, a successful station-based auto-dissemination approach that reduces the mosquito population will reduce the number of ovipositions by the mosquito population (O). Assuming that the number of potential breeding sites (H) remains constant within the habitat, fewer females will result in fewer ovipositions, which is predicted to reduce the coverage of breeding sites (S1 Fig.). Efficacy differences between field sites observed in prior field trials of a station-based approach were suggested that differing abundance of mosquitoes between the sites as a possible explanation [19,20].

The model predicts that a method offsetting the above predicted limitations would be to artificially sustain mosquito activity through the release of mosquitoes. By maintaining high mosquito activity, the delivery of the larvicidal agent can continue. Clearly however, sustaining a population through the release of female mosquitoes, which bite and could transmit pathogens, would be an unacceptable approach. However, the introduction of adult male mosquitoes, which do not bite or transmit pathogens, is feasible. There are multiple vector-control strategies that are based on the repeated, inundative release of adult male mosquitoes, including Sterile Insect Technique (SIT) and newer strategies based on transgenic mosquitoes (*e.g.*, RIDL) [35].

Furthermore, because the males are reared artificially and released, the ADAM approach would provide opportunity for their direct treatment with the larvicide, prior to their release, rather than relying on dissemination stations to contaminate males. Direct treatment in a controlled environment can permit a more uniform and standardized application of the larvicide, relative to a station-based approach.

Empirical Examination of the ADAM Approach

Effect of PPF dust on adult male survival. Critical to the proposed ADAM approach, the laboratory-reared male adults must remain competitive after treatment with the insecticide. To examine for an acute effect on survival, three replicate groups of males were divided and either dusted with PPF (treatment) or not dusted (control). Following treatment, mosquitoes were held in the laboratory and observed for male mortality. As illustrated in Fig. 2, comparing treatment and control groups, male survivorship was not observed to differ (Kaplan-Meier, Log-Rank, $p > 0.32$).

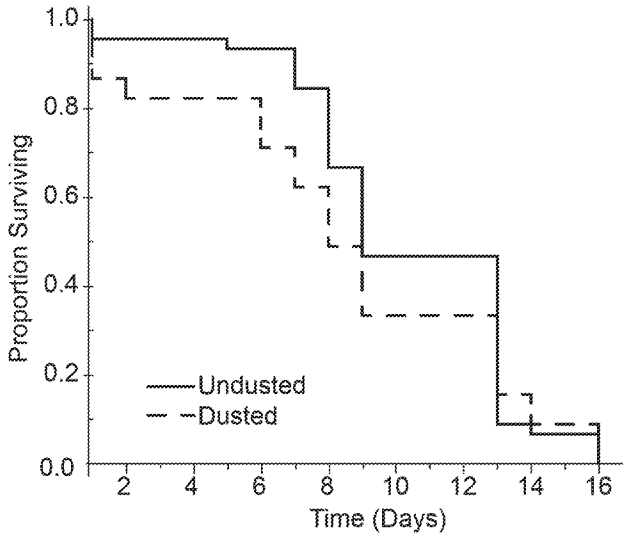

Figure 2. Survivorship of male *Ae. albopictus* treated with Pyriproxyfen/Dayglo dust compared to untreated males.

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Transfer of PPF from males to females and ovisites. In an additional experiment, field cages were used to examine the ability of PPF-treated males to interact with and cross contaminate females. PPF-treated males were introduced into field cages. Untreated females, *i.e.*, not PPF-treated, were introduced into cages also. Field cages included ten oviposition sites ('ovisites'), five of which were covered with screen to prevent mosquito entry.

Five days after mosquito introduction into cages, adults and ovisites were removed from the cages. Ovisites were visually examined for PPF powder residue (Fig. 3). No PPF powder was observed in the screen-covered ovisites, *i.e.*, from which mosquitoes were excluded (n=15 cups). In contrast, PPF powder was observed in all but one of the unscreened cups, *i.e.*, in one cage replicate, no powder was observed in one of the five unscreened cups. Dead adults (n=5) were observed in two of the unscreened cups (Fig. 3) and none of the screened cups.

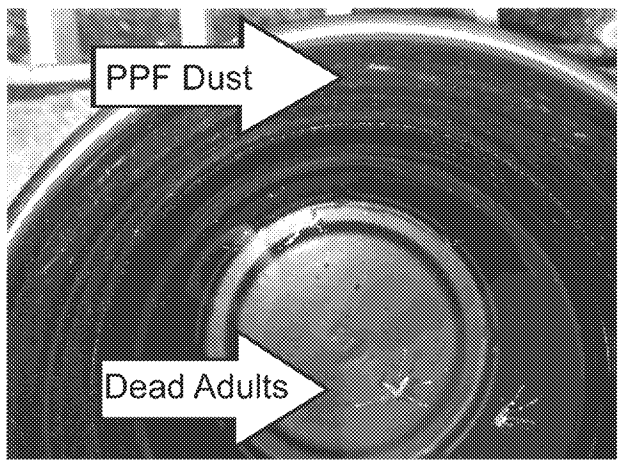

Figure 3. An example of an ovisite removed from a field cage trial. Arrows point to pyriproxyfen dust on the sides of the cup and to two dead adult *Ae. albopictus* floating in the water.

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Adults removed from cages were bioassayed for toxicity against *A. albopictus* larvae. The bioassay results demonstrate significantly higher mortality of larvae exposed to males and females removed from field cages, relative to the negative control bioassays ($p < 0.0001$, Kruskal-Wallis). The survivorship of larvae exposed to adult females from field cages was 35.6±6.1%, mean±std err (Fig. 4). In contrast, higher survival of immatures was observed in bioassays not receiving an adult from field cages, *i.e.*, negative control, was 87.5±4.7%. Adult males removed from cages and introduced into immature bioassays resulted in 3.6±3.6% immature survivorship, which was not significantly different from the positive control assays (0±0% immature survivorship). No difference was observed between the three field cage replicates ($p > 0.2$, Kruskal-Wallis).

In addition to the living adults removed from field cages, five dead males were recovered from unscreened cups, floating in the water. Bioassays with the latter five males were 100% lethal to exposed larvae in bioassays, with no larvae surviving in bioassays (n=5).

As described above, PPF powder residue was observed in the majority of the unscreened ovisites at the end of the experiment. Therefore bioassays were conducted to examine for PPF residue introduced into the ovisites. For assays, each cup was separated into three components: water, paper lining and cup. Water samples removed from cups were tested by bioassay, with two samples per cup. As shown in Fig. 5, high immature survivorship (92.5±2.1%) was observed in bioassays of water from screened cups, *i.e.*, mosquitoes excluded. In contrast, no larvae survived in assays of water samples removed from unscreened cups. Comparison with the control groups show no difference between the unscreened and positive control (Fig. 5). The results from screened cups did not differ significantly from that of the negative control bioassays. Similar to the results of the water bioassays, assays of the germination paper lining and the surface of the unscreened cups were highly lethal to larvae in bioassays, not different from

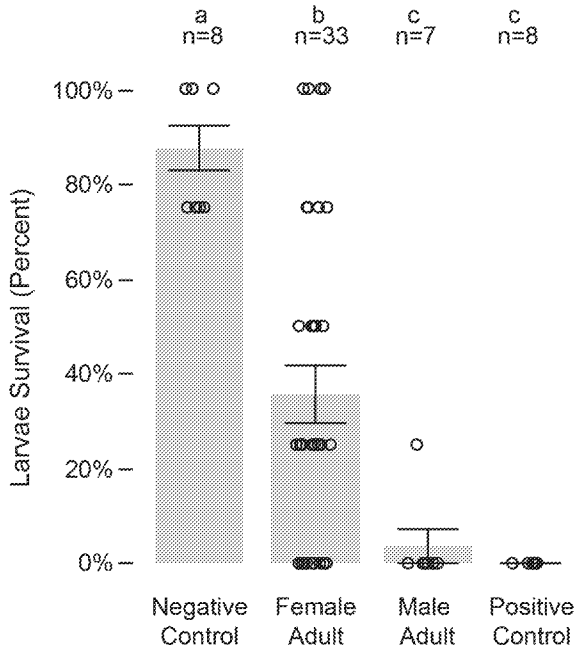

Figure 4. Adults removed from field cages were examined for insecticidal activity using immature bioassays. Letters above the bars indicate significant differences ($p < 0.01$, Wilcoxon). The number of replicates is shown above each column. Bars show standard errors.

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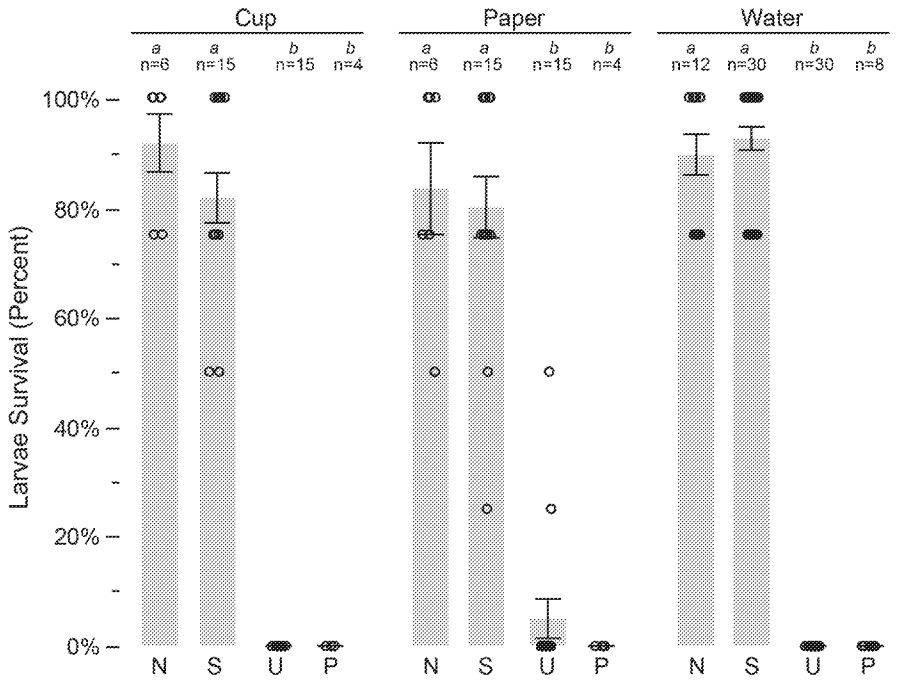

Figure 5. Oviposition cups removed from field cages were separate into three different components (*i.e.* cup, paper lining, and water), and each was examined for insecticidal activity using immature bioassays. For each component, screened (S) and unscreened (U) cups are compared, along with the negative (N) and positive (P) controls. Letters above the bars indicate significant differences ($p < 0.01$). The number of replicates is shown above each column. Bars show standard errors.

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that observed in the positive control group (Fig. 5). In contrast, high survivorship was observed in assays of the screened cups, similar to that of the negative control group.

Field trial of the ADAM approach. Based on field cage results, open releases of PPF-males were conducted, to examine the ability of males as PPF carriers in the field. At the treatment site (Fig. 6A), ovisites and BG traps were positioned at variable distances from a release point. Additional ovisites were monitored at two untreated sites >4 km from the treatment site. Following nine weeks of pre-introduction population monitoring, an average of 4,500 PPF-treated males/week were introduced at the Treatment site (Fig. 7A). An immediate toxic effect was observed in water sampled from ovisites from the Treatment site (Fig. 7B), while bioassays of water from the Untreated site continued to result in good survival (>80%; Fig 7B). Toxicity at the Treatment site persisted for the duration of the release period.

No immediate impact on the adult population was observed following the introduction of PPF-dusted males. However, a decline in the adult population at the Treatment site was observed (Fig. 7A), beginning approximately four weeks after the initial ADAM male introduction, *i.e.*, beginning at Week 14. In contrast, a decline was not observed in the populations at the Untreated sites; instead these populations continued at densities similar to that observed during the pre-introduction period. Repeated Measures ANOVA shows no significant SITE effect, but there were significant effects by WEEK, $F_{(6,36)} = 5.8$; $p < 0.0002$ and the WEEK*SITE interaction, $F_{(6,36)} = 6.0$; $p < 0.0002$. Linear regression of the two Untreated sites during Weeks 14–20 were either non-significant or significantly positive, *i.e.*, increasing population (S2 Fig.). At the Treatment site, however, the regression during the same period was significantly negative following male introduction (S2 Fig.). At Week 21, the ambient air temperature dropped to 6°C, and no additional mosquitoes were collected at any of the sites.

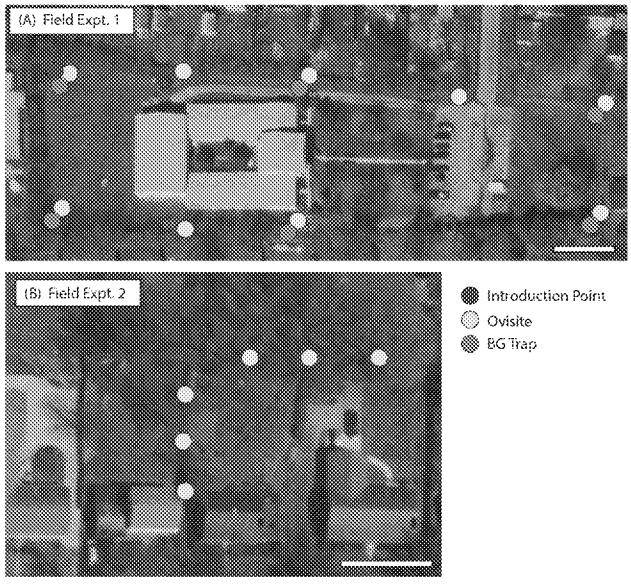

Figure 6. Field sites for male introduction experiments in Lexington, Kentucky. (A) Field Experiment 1 Site consisted of a single point introduction site (red circle), six BG trap sites (blue circle) and nine ovisites (yellow circles). (B) Field Experiment 2 Site consisted of a single point introduction site and six ovisites. Images are from http://datagateway.nrcs.usda.gov/GDGHome.aspx. Bars = 60ft.

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Examining adult males as direct carriers. An important characteristic of the ADAM approach would be the ability of males to deliver insecticide directly to breeding sites, in the absence of females. In the preceding study, because both females and males were present, the PPF contamination could have resulted from indirect contamination by males, *i.e.*, males cross-contaminate females, and the females subsequently contaminate breeding sites. Therefore, to examine the ability of males to act as 'direct carriers,' delivering PPF directly to breeding sites, a second field study was performed in early spring, before the indigenous population was observed.

Similar to the prior field experiment, ovisites were placed at varying distances away from a release point (Fig. 6B), and water was sampled weekly and assessed in larval bioassays. An additional array of ovisites was deployed and tested at an Untreated site, which was >4 km from the Treatment site. Water samples were drawn for two weeks prior to male introduction and two weeks during male introduction. To monitor for the appearance of the indigenous population, landing counts were performed weekly at both the Treated and Untreated sites.

In the two week Pre-release period, prior to male introduction, good immature survival (89.6±19.4% survival, n=24) resulted in bioassays of water sampled at both sites (Fig. 8), with no difference observed between the Treated and Untreated sites ($p > 0.15$, Wilcoxon). Subsequently, 6,300 and 5,100 PPF-treated males were introduced at the Treatment site in Weeks 3 and 4, respectively. During the two-week male introduction period, a significant difference was observed between the Untreated and Treated sites (Fig. 8). Bioassays of water sampled closest to the release point were completely lethal to immatures (0% survival). As shown in Fig. 9, a significant correlation was observed between bioassay lethality and distance from the release point during the introduction period, but not during the pre-introduction period. In contrast, high survival continued in assays of water from the Untreated site throughout all four weeks (Fig. 8). No females, *e.g.*, indigenous population, were observed during the study.

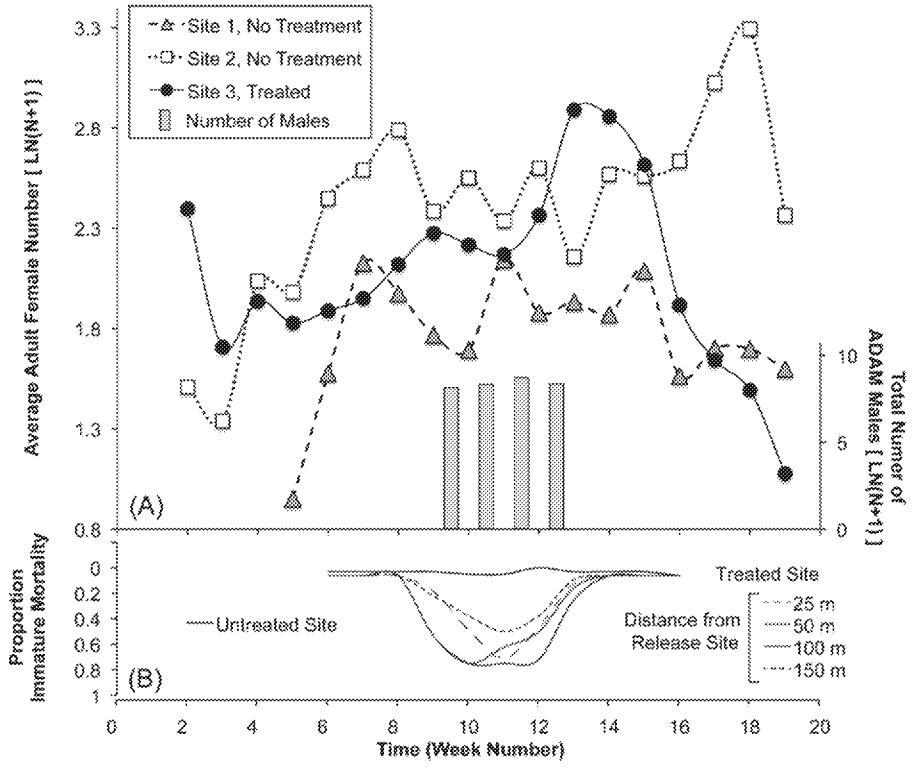

Figure 7. Results of Field Trial 1. (A) *Ae. albopictus* adults are monitored using BG traps at three sites. Grey bars indicate introductions of PPF-dusted males at Site 3 only. Beginning after Week 13, a consistent population decline is observed at Site 3, which is not observed at the untreated sites. Lines show a three-week moving average for the adult collections. (B) Bioassays of artificial oviposition sites show increased larval mortality up to 150m from the Site 3 release point. In contrast, low mortality is observed in bioassays of ovisites within untreated areas.

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Discussion

The primary objective of this study was to assess the ADAM mosquito control strategy, which combines components of both autocidal and auto-dissemination approaches. Here, we have examined the ADAM approach against *Ae. albopictus*, a globally invasive species and important pathogen vector. The results demonstrate that, under the conditions tested here, *Ae. albopictus* males treated directly with PPF do not suffer a measurable decrease in longevity, relative to untreated males. In field cage trials, PPF dust was transferred from males to females and to larval breeding sites, at levels adequate to reduce or eliminate immature survival. Field trials show that the introduced males can quickly disseminate PPF, both in the absence and presence of female *Ae. albopictus*. Under the conditions tested, the PPF persists for at least six days after the males were dusted.

Bioassay results show that females in the field cage experiment become contaminated with PPF dust. Because the adult females were not treated with PPF, the contamination of females necessarily resulted from cross-contamination by males. An obvious opportunity for horizontal transfer between males and females is via coupling during mating and mating attempts. This type of transfer would be similar to that described in previous work with auto-dissemination stations [18]. Indirect transfer to females, via resting surfaces, *e.g.*, water or ovisite sides (Fig. 3), is an additional potential route for female contamination with PPF.

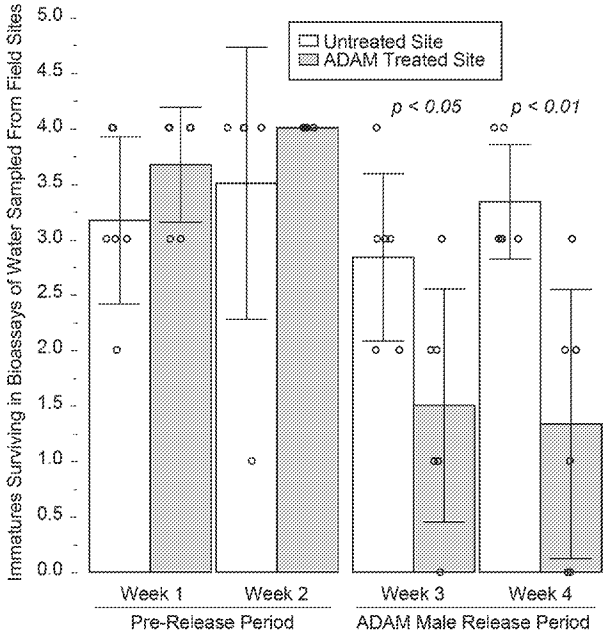

Figure 8. In the absence of an indigenous *Ae. albopictus* population, *i.e.*, Field Trial 2, increased immature mortality is observed following the introduction of PPF-treated males. During the pre-release period, good larval survival is observed in bioassays of water sampled from ovisites at both the Untreated and Treated sites. Following male introductions, reduced survival is observed at the Treated site only. Significant differences are indicated above the columns (Wilcoxon). Bars show standard deviation.

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Early season field trials demonstrate that male *Ae. albopictus* can directly deliver PPF to ovisites, in the absence of females, and at levels that are lethal to immatures. Male mosquitoes are understudied in general, and their behavior relative to breeding sites has not been well defined. Presumably, male *Ae. albopictus* may visit ovisites for hydration or as favorable, humid microhabitat refuges. Or perhaps the male behavior of visiting ovisites can be adaptive, by increasing the frequency of female encounters and mating opportunities. This represents an area for additional study.

An ability of males to directly deliver compounds to breeding sites can provide useful functionality, relative to auto-dissemination approaches that rely upon indigenous mosquitoes to communicate the active ingredient from the introduced station. By using laboratory-reared, male mosquitoes as vehicles, the ADAM approach can be deployed in areas that have relatively low indigenous mosquito densities. As an example, our results show that introduced males can intoxicate potential breeding sites, before the seasonal emergence of the indigenous population. This can allow anticipation of a seasonal increase, which can accelerate application, relative to an approach that is dependent upon the indigenous population. Direct treatment of laboratory-reared males allow for uniform application of the pesticide under controlled conditions. There is no need to deploy or maintain auto-dissemination stations.

The results show that, in addition to direct transmission of lethal compounds, males can cross-contaminate females, at dosages that are lethal to developing mosquitoes. The subsequent transmission to breeding sites by females is similar to that of traditional, station-based auto-dissemination approaches. Female transmission can be an important component, because ovipositing females can visit multiple larval breeding sites, treating each with a toxic dose. Additional downstream work, ideally with observations occurring in the field, will help to better define the transfer pathways and their relative importance.

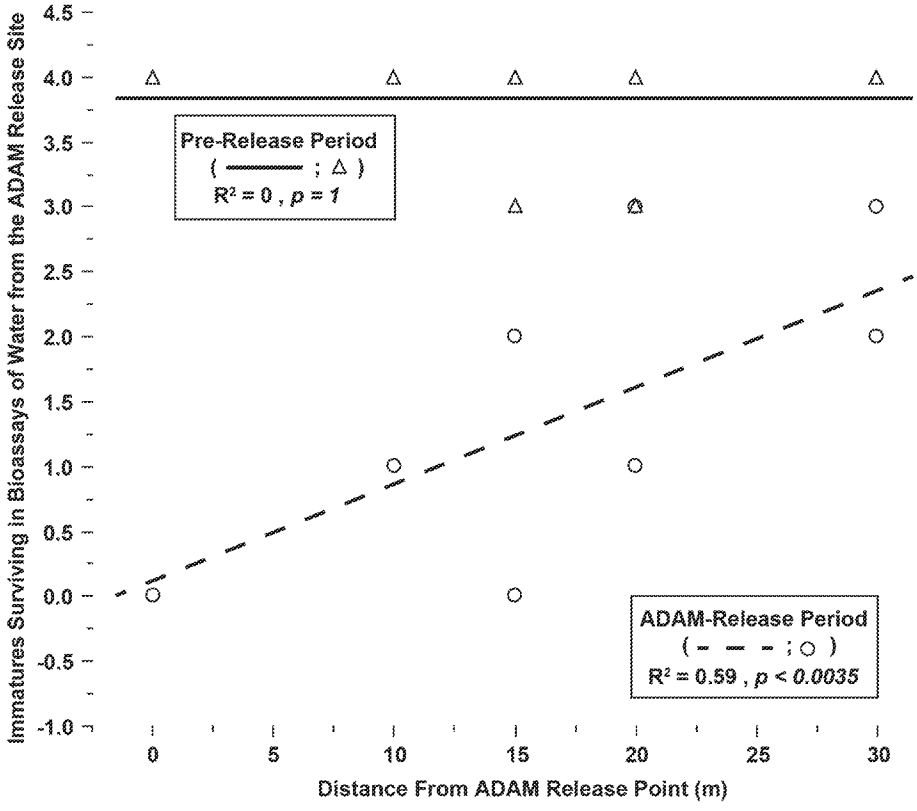

Figure 9. Immature survivorship in bioassays is correlated with distance from the introduction point in Field Trial 2, but only during the PPF-male introduction period. A bivariate fit of Survival versus distance is significant, but only during the two weeks of male introductions.

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The results support the continued development of the ADAM approach and open field trials in which PPF-treated *Ae. albopictus* males are released to directly contaminate *Ae. albopictus* females and breeding sites. While here we have tested the approach against *Ae. albopictus*, similar work can examine the utility of this approach against additional medically important species of mosquitoes, *e.g.*, *Ae. aegypti*, *Culex pipiens*, and *Anopheles spp*. Because different species can share common breeding sites, *e.g.*, it is common to find both *Ae. aegypti* and *Ae. albopictus* in the same larval habitats [36], an approach based on the introduction of PPF-treated *Ae. albopictus* males can affect populations of both *Ae. albopictus* and *Ae. aegypti*.

PPF-treated or not, an ADAM approach based on an exotic species is less likely to be adopted as a control tool. Therefore, adapting the ADAM approach to indigenous species can facilitate its use in a broader range of geographic areas. We note that the species used in an ADAM approach need not be mosquitoes or males necessarily. The key decision factors in species selection will include that the released insects (1) should not cause harm, *e.g.*, bite or transmit pathogens or be an agricultural pest, (2) should deliver, directly or indirectly, the insecticide to the larval breeding sites of the targeted insect species, and (3) should be colonized and relatively easy to manage and rear.

Similar to other insecticidal approaches, the issue of effects to non-target organisms must be considered, *e.g.*, potential for affecting larval competition. The amounts of PPF used via the ADAM approach are likely to be similar to that of station-based auto-dissemination strategies, and likely to be less than that of a human applied, broadcasting approaches. While the use of

alternate active ingredients in the ADAM approach can be envisioned, the characteristics of pyriproxyfen make it an interesting candidate. These features include its high toxicity to immature mosquitoes, low toxicity to adult mosquitoes, a substantial amount of prior research and environmental assessment, and its classification as a low risk insecticide [29]. The non-target organism toxicity characteristics of PPF compare favorably with many other approved insecticides [37-40]. PPF effects are mainly at the pupal stage, when mosquitoes are not feeding and resource competition is less. This late-acting effect can be advantageous in mitigating density-dependent effects, which can offset the impact of earlier-acting compounds [41,42]. An additional feature of PPF that may be examined in future ADAM-related work are its effects on female fertility and male spermiogenesis [23,43-45]. Specifically, PPF contamination of females can reduce fertility of females and males, which can negatively impact the population in addition to the pupacidal effect of PPF [46].

We envision that an ADAM approach would be one component of an integrated vector management strategy. The strengths of the ADAM approach would be that of (1) 'self-delivery,'similar to other autocidal approaches, and (2) the ability of mosquitoes to find/treat cryptic breeding sites, similar to other auto-dissemination approaches. The small dosages delivered by the ADAM approach are less likely to affect large-volume pools, ponds, etc. But the latter are a strength of existing, traditional larviciding strategies. Adulticiding will continue to be needed for quick knock down of the adult mosquito population, but appropriate operational timing can allow for the integration of many adulticiding approaches with the ADAM approach, and alternation of different active ingredients can help to mitigate insecticidal resistance.

Similar to additional autocidal approaches, the *Ae. albopictus* ADAM approach will require large-scale production, *i.e.*, 'mass rearing,' of mosquitoes for release. This type of mass rearing operation is developed already and in use with other autocidal approaches, including Sterile Insect Technique (SIT) [47-49]. Furthermore, the potential benefit of PPF treatment to 'boosting' autocidal approaches has been highlighted previously [50].

Here we have examined a new approach against mosquitoes, which combines components of both auto-dissemination and autocidal methods. Clearly, there is need for additional, larger field trials, conducted within different ecological contexts and culminating in community-randomized controlled trials. Relative to station-based auto-dissemination approaches, attractive features of the ADAM approach include (1) the ability to directly apply larvicidal compounds and thereby avoid complicating variation caused by mosquito self-treatment and variable environmental conditions; (2) an ability to regulate the size and location of treated-male introductions, expanding the utility to areas where mosquito populations are low, *e.g.*, early in the season; and (3) avoiding the requirement of placing, maintaining and recovering dissemination stations.

The classification of PPF as a low risk compound, its relatively low environmental impacts [23,29], its residual activity (4 months in water) [25], and absence of resistance in mosquito populations [26] help to make PPF an attractive candidate for the ADAM approach. The susceptibility of multiple species of Aedes, Culex and Anopheles [51] and the fact that multiple mosquito species can share the same breeding sites [12] allow this approach to be extended to additional, medically important systems, with the potential to impact dengue, malaria, filariasis, chikungunya and additional mosquito-borne pathogens.

Supporting Information

S1 Fig. Model predictions are that the success of the auto-dissemination approach depends on mosquito activity. Therefore the model (Equation 1) predicts that an auto-dissemination

approach that is reliant on indigenous mosquitoes will (1) be relatively ineffective in areas of low mosquito activity and (2) can become a victim of its own success. With fewer mosquitoes, fewer ovipositions (O) will occur. Assuming that the number of potential breeding sites (H) and insecticide potency (Ω) remain constant, fewer mosquitoes will result in fewer ovipositions/habitat and lower coverage of breeding sites (C_h). This pattern is consistent despite the durability of the pesticide (U) [19].
(TIF)

S2 Fig. Simple linear regressions of the adult female collections over time by site. Untreated Sites 1 and 2 are non-significant ($p > 0.9$) and significantly positive ($p < 0.049$), respectively. Site 3, which was treated with ADAM males, declined significantly ($p < 0.0001$) following treatment.
(TIF)

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Author Contributions

Conceived and designed the experiments: JWM CLB SLD. Performed the experiments: JWM CLB SLD. Analyzed the data: JWM CLB SLD. Contributed reagents/materials/analysis tools: JWM CLB SLD. Wrote the paper: JWM CLB SLD.

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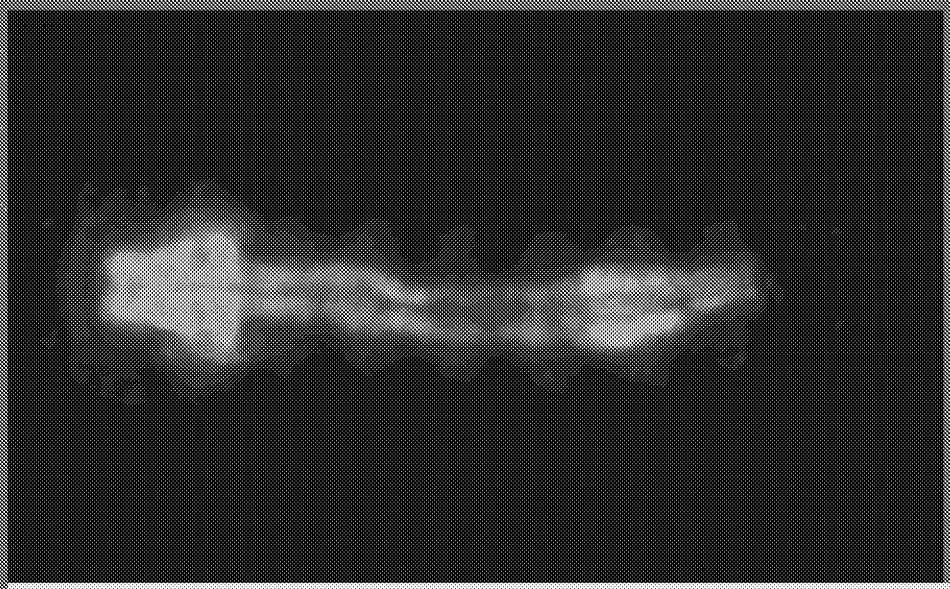

Guidance framework for testing of genetically modified mosquitoes

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Glossary

Alleles – different forms of the same gene.

Area-wide control – methods of reducing pest damage whose effectiveness depends on application over large expanses. This contrasts particularly with personal protection, for example as provided by bed nets and repellents.

Biosafety committee – group responsible for implementing policies and guidelines related to use of potentially hazardous biological agents, including but not limited to infectious agents, human materials, and recombinant DNA studies. This group ensures that research involving these agents does not endanger researchers, laboratory workers, human research subjects, the public or the environment.

Cartagena Protocol on Biosafety – an international agreement dealing with the safe handling, transport and use of living modified organisms (LMOs) resulting from modern biotechnology. See: http://bch.cbd.int/protocol/

Clinical disease incidence – the number of new clinical cases per unit of time for the at-risk population. This is typically determined by voluntary reporting of symptoms or community-based active case detection followed by a laboratory diagnosis test.

Cluster randomized trials – trials that group individuals into clusters, such as residents of particular villages or urban neighbourhoods. Each cluster is assigned randomly an experimental treatment such as a placebo or drug, or, in the case of genetically modified mosquitoes (GMMs), releases may be in one set of clusters and not in another.

Community engagement – practices undertaken to inform stakeholders about the diseases and vectors of interest and goals of a proposed research study or intervention trial, and to understand their perspectives and reaction.

Confinement – utilization of measures that seek to prevent unplanned or uncontrolled release of organisms into the environment. This may involve physical confinement (sometimes termed "containment") within a large cage that simulates the disease-endemic setting while minimizing the possibility of escape and/or ecological confinement by geographic/spatial and/or climatic isolation.

Declaration of Helsinki – a set of ethical principles for the medical community regarding human experimentation, issued by the World Medical Association.

Deployment – implementation of GMM technology as part of a national or regional programme for vector control.

Drive (also called gene drive) – a mechanism that increases the transmission of a transgene in a population above that which would be expected based on Mendelian inheritance. The increase is reflected in the excess proportion of progeny that carry the transgene.

Ecosystem – a biological system composed of a community of organisms and the nonliving environment with which it interacts.

Endemic – a situation in which disease is present continuously at some level in an area.

Endpoint – an event or outcome that can be measured objectively to determine whether the intervention being studied has the desired effect.

Entomological inoculation rate (EIR) – a measure of the degree of infection risk that a human population is exposed to for a particular disease, as determined by assessing the vector mosquito population. It is described by the frequency of infectious mosquitoes feeding upon a person within some unit of time, such as per day or year.

Epidemic – an increase in incidence and prevalence of disease affecting many people rapidly and extensively and above normal levels in an area, but not continuously present at such levels.

Ethics – an activity or inquiry intended to shed light on the correctness or justifiability of a given course of conduct.

Ethics committee (also called institutional ethics committee, institutional review board or ethical review board) – a group charged with providing oversight for biomedical and behavioural research involving humans, with the aim to protect the rights and welfare of research subjects.

Ethical review board – see *Ethics committee.*

Fitness – description of the ability to both survive and reproduce, and is equal to the long-term average contribution to the gene pool by individuals having a particular genotype or phenotype. If differences between alleles of a given gene affect fitness, then the frequencies of the alleles will change over generations, the alleles with higher fitness become more common.

Gene – a segment of DNA that contains information required by cells for synthesis of a product.

Gene flow – the movement (expressed as increase in frequency) of genes or alleles into a population from one or more other populations.

Genetically engineered mosquitoes – see *Genetically modified mosquitoes.*

Genetically modified mosquitoes (GMMs) – also called genetically engineered mosquitoes, transgenic mosquitoes, or living modified mosquitoes – mosquitoes that have heritable traits derived through use of recombinant DNA technology, which alter the strain, line, or colony in a manner usually intended to result in reduction of the transmission of mosquito-borne human diseases – see also *Genetically Modified Organism*. GMM is also likely to be characterized by introduced heritable marker traits to facilitate monitoring upon release into the environment and in some cases may include only such markers, as for population biology studies.

Genetically modified organism (GMO) – also called living modified organism – any organism that has in its genome novel DNA of endogenous, exogenous, or mixed origin that was made using modern recombinant DNA technology. Although successive selective breeding of strains of organisms with naturally-occurring allelic variations also results in strains with genotypes different from the natural population, these are excluded from this definition.

Genotype – the genetic constitution of an organism.

Good clinical practice (GCP) – an international quality standard for trials involving human subjects, including protection of human rights, assurance of safety and efficacy and standards on conduct of clinical trials. See: http://www.emea.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002874.pdf

Hazard – an event, activity or other cause of a negative consequence or impact identified in a risk analysis.

Horizontal gene transfer (HGT) – heritable transfer of a functional genetic element from one organism to another without mating, most often relating to genetic exchange between different species.

Infection incidence – the rate at which new infections occur during the specific period of time.

Informed consent – the process intended to ensure that human subjects who will be observed or involved in a research activity are fully and explicitly advised of all risks, costs or inconveniences they may bear as a result of participating as a research subject, and voluntarily agree to accept or bear those risks and costs.

Institutional ethics committee (IEC) – see Ethics committee.

Institutional review board (IRB) – see Ethics committee.

Integrated vector management (IVM) – a rational decision-making process for the effective and efficient use of a combination of available resources in the management of vector populations, so as to reduce or interrupt transmission of vector-borne diseases. See: http://www.who.int/malaria/vector_control/ivm/en/

Living modified mosquitoes – see *Genetically modified mosquitoes.*

Mark-release-recapture – a method used to estimate population size of free-living animals, including mosquitoes, and to study population survival and dispersal in space and time. A portion of the mosquito population under study is captured, marked (usually with fluorescent powders) and released. A portion of the population into which they were released is captured later and the number of marked mosquitoes within the sample is counted. The proportion of marked mosquitoes in the second sample allows estimation of the total number of animals in the whole population.

Non-target organism – any organism that is not a direct target of an intended intervention. For GMM the direct target organism is other mosquitoes of the same species in the wild population.

Nuremberg Code – an ethics code that serves as a basis for bioethical principles ensuring the rights of human subjects in medical research.

Off-target effects – the outcomes of actions that are not directed to the purpose of the action, whether anticipated or not, possibly affecting either target or non-target organisms. Off-target effects may have negative, neutral or positive impacts on the intended purpose.

Pathogen – an organism that causes disease. In dengue infection, the pathogen is a virus. In malaria infection, the pathogen is a unicellular parasite.

Penetrance – the frequency at which a trait is expressed in individuals carrying a particular gene associated with the trait.

Pharmacovigilance – the process of collecting, monitoring, researching, assessing and evaluating information on the long-term adverse effects of medicines.

Phenotype – the observable characteristics of an organism, based on genetic and environmental influences.

Population regulation – maintenance of a population around or near an equilibrium level, such as by density-dependent factors.

Population replacement – strategies that target vector competence with the intent to reduce the inherent ability of individual mosquitoes to transmit a given pathogen.

Population suppression – strategies that target vector "demography" with the intent to reduce (suppress) the size of the natural mosquito population to the extent that it would not be able to sustain pathogen transmission.

Prevalence of infection – the frequency of infection within a population at any given time.

Refractoriness – a condition in which the mosquito is intrinsically unable to support the development of a pathogen to an infective stage or to a point of sufficient abundance such that the mosquito cannot transmit disease.

Regulation – an official rule to manage the conduct of those to whom it applies, usually developed from legal interpretations of legislation and implemented by government ministries or agencies.

Regulatory agency (also called regulatory authority, ministry, regulatory body, or regulator) – a public authority or government entity responsible for exercising authority over some area of activity in a supervisory capacity.

Risk – an objective measure of the product of the likelihood and consequences of a hazard, defined within a prescribed set of circumstances. Risk is often described as a probability distribution of a set of consequences over a defined time period.

Risk analysis – the process comprised of risk identification, risk assessment, risk management and risk communication.

Risk assessment – a methodological approach to define and characterize hazards, and to estimate the exposure or likelihood of each hazard occurring as well as the potential adverse impact of the hazard (harm).

Risk management – the process of identifying and implementing measures that can be expected to reduce risk to an acceptable level.

Risk communication – the process through which risk concerns and risk tolerance is articulated by relevant stakeholders and results of risk assessment and risk management are communicated to decision-makers and the public.

Self-limiting – GMM approaches where the genetic modification will not pass on indefinitely through subsequent generations.

Self-sustaining (also called self-propagating) – GMM approaches where the heritable modification is spread and maintained indefinitely through the target population.

Sterile insect technique (SIT) – the inundative release of factory-produced sexually sterile insects into wild native insect populations so that there is a high ratio of sterile males to wild females. Sterilization is usually accomplished using radiation or chemicals. The effect is population suppression, and the effort is most effective when continual and over large areas to reduce the effects of fertile immigrants. Release only of males is preferred although release of both sexes has also been effective. SIT has been applied most widely against agricultural pests.

Traits – phenotypes that result from single or multiple genes and their interactions with the environment.

Transboundary movement – movement across national, state or other political lines of demarcation.

Transgenic mosquitoes – see *Genetically modified mosquitoes.*

Vector mosquitoes – mosquitoes that are able to transmit a disease-causing pathogen.

Abbreviations

APHIS	US Animal and Plant Health Inspection Service
CBD	Convention on Biological Diversity
CPB	Cartagena Protocol on Biosafety
CSO	Civil society organization
DNA	Deoxyribonucleic acid
EA	Environmental assessment
EFSA	European Food Safety Authority
EIA	Environmental impact assessment (also known as a strategic environmental assessment or environment impact statement
EIR	Entomological inoculation rate
EIS	Environmental Impact Statement under the US National Environmental Policy Act
ERA	Environmental risk assessment
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FDA	US Food and Drug Administration
FFDCA	US Federal Food Drug and Cosmetic Act
FIFRA	US Federal Insecticide and Rodenticide Act
FNIH	Foundation for the National Institutes of Health
GM	Genetically modified
GMM	Genetically modified mosquito
GMO	Genetically modified organism
IPPC	International Plant Protection Convention
ISPM	International Standards for Phytosanitary Measures
LMO	Living modified organism
NAPPO	North American Plant Protection Organization
NEPA	National Environmental Policy Act (USA)
NTO	Non-target organism
RA	Risk assessment
RM	Risk management
SOP	Standard operating procedure
SPS	WTO Agreement on the Application of Sanitary and Phytosanitary Measures
SIT	Sterile insect technique
UNDP	United Nations Development Programme
USDA	US Department of Agriculture
WHO	World Health Organization
WHO-TDR	World Health Organization Special Programme for Research and Training in Tropical Diseases
WTO	World Trade Organization

Foreword

Vector-borne diseases are endemic in more than 100 countries and affect approximately half of the world's population. Many types of arthropods may serve as disease vectors, but this guidance focuses particularly on mosquitoes. Mosquitoes transmit several diseases of major global public health importance, including malaria and dengue fever.

Despite ongoing and intensive control efforts, malaria and dengue continue to exact a huge public health toll. Malaria is considered the world's most important parasitic infectious disease. Estimates of malaria-related deaths in 2010 range from 655 000 (WHO, 2011) to over 1.2 million (Murray et al., 2012), with the majority of deaths occurring among African children under five years of age. The international Roll Back Malaria partnership has pledged a goal to "eradicate malaria worldwide by reducing the global incidence to zero through progressive elimination in countries."[1] Yet it is acknowledged widely that this goal will not be met without new tools (Greenwood et al., 2008; Mendis et al., 2009; Alonso et al., 2011; Alonso & Tanner, 2013). An estimated 2.5 billion people live in areas where dengue viruses can be transmitted. Despite a plan adopted by the Pan-American Health Organization (PAHO) and its Member States to eventually eradicate *Aedes aegypti*, the main vector of dengue in the Americas (PAHO, 1997; 1998), dengue continues to plague countries in Latin America, as well as Asia and Africa. In 2013, the estimated global burden of dengue was revised upward to 390 million infections per year (Bhatt et al, 2013). WHO recently called dengue the most important mosquito-borne viral disease with an epidemic potential in the world, citing a 30-fold increase in the global incidence of dengue during the past 50 years and recognizing that the human and economic costs are staggering. WHO further acknowledged that innovations in vector control deserve more attention as playing a key part in reducing transmission and disease burden.[2]

Attacking mosquito vectors is one of the most effective ways to reduce the transmission of disease in endemic areas. Application of mosquito population reduction methods was central to successful elimination of malaria transmission in Italy and the United States of America in the early 20th century (Kitron & Spielman, 1989) and, transiently, of dengue in the Americas in the early 1960s (Pinheiro & Corber, 1997). Vector-targeted approaches remain a mainstay of current disease-control practices. However, given the magnitude of ongoing malaria and dengue incidence, current efforts clearly are insufficient to meet the need. Moreover, dependence on a limited number of insecticides for vector control increases the risk that mosquitoes will develop resistance, as is now being widely reported (Butler, 2011). In 2012, WHO confirmed that insecticide resistance is being reported in two-thirds of countries with ongoing malaria transmission, and that resistance affects all major vector species and classes of insecticide (WHO, 2012).

In considering the potential of new technologies to address the unmet needs of mosquito control, it is necessary to evaluate the benefits and risks in the context of the current situation. The potential public health benefit of practical and effective new tools to reduce or even eradicate diseases such as malaria and dengue is clear and widely recognized. Both the risks incurred by testing new, and

[1] Roll Back Malaria: http://www.rollbackmalaria.org/rbmvision.html, accessed 25 May 2014.
[2] Second WHO report on neglected tropical diseases: http://www.who.int/neglected_diseases/9789241564540/en/, accessed 25 May 2014.

unproven strategies and the risks to human health and the environment posed by maintaining the status quo, which include ongoing disease and use of broad spectrum insecticides, should be taken into account in decision-making.

For more than two decades, scientists have been working to harness the promise of molecular biology to develop genetically modified mosquitoes (GMMs) for use as public health tools to prevent the transmission of these diseases. Several of these genetic technologies are now advancing to field testing. The introduction of molecular biology techniques represents the next step in a progression that builds on the widespread success of programmes employing release of radiation-sterilized insects to control the Mediterranean fruit fly (Med fly) and other insect pests affecting plants and animals, a process known as Sterile Insect Technique (Dyck, Hendrichs & Robinson, 2005). Radiation- and chemo-sterilization methods also have been applied to mosquitoes (Dame et al., 2009), but they pose several difficulties that might be overcome using genetic modification technologies. Recent advances in the development of GMMs have raised hopes for the availability of new, potent and cost-effective tools to aid in the fight against malaria and dengue. Data on which to base evaluation of the protective potential of GMMs can only be collected through testing, including testing under the natural conditions in which the technology would be utilized. Without the ability to conduct careful and stepwise testing, no new technology can be brought to fruition for the public good. However, given the novelty of GMMs, concerns have been raised about the need for thorough, thoughtful and transparent preparation for and conduct of field trials (Reeves et al., 2012) and frameworks for environmental risk assessment (RA) have been produced at various levels (examples are provided in Section 3. Biosafety, and in David et al., 2013).

Since 2001, scientists involved in this research have, with the support of TDR, the Special Programme for Research and Training in Tropical Diseases (WHO-TDR) and other funders, gathered periodically to consider issues relevant to testing and implementation of genetically modified vectors. Through such discussions, broad agreement has been reached within the scientific community on two tenets, which thus far have been observed.

- First, field-testing should begin with release of sterile or otherwise self-limiting modified male mosquitoes in order to gain experience with the technology under circumstances where its effects can be controlled by halting releases (Benedict & Robinson, 2003). Field releases of GMMs carried out to date have focused on the testing of non-replicating, functionally sterile, males (which do not bite).
- Second, testing of modified mosquitoes incorporating gene drive should begin under physical confinement (Alphey et al., 2002; Benedict et al., 2008). No GMMs designed to replicate and spread the modification to wild-type mosquitoes have yet been tested outside of the laboratory.

As the research progresses, a need has been expressed both within the scientific community and by the public for additional standards and guidance. WHO-TDR and the Foundation for the National Institutes of Health (FNIH) co-sponsored a technical consultation meeting in 2009 to assess current progress and future development of genetically modified mosquito technologies. The meeting was attended by participants from around the world with expertise in molecular biology, medical entomology, ecology, regulatory requirements, ethical, social and cultural issues, as well as staff from WHO, FNIH and other research funders WHO-TDR, 2010). Participants recommended the establishment by WHO and FNIH of a working group to develop a comprehensive guidance framework to provide quality standards for assessing the safety and efficacy of genetically modified

mosquitoes and addressing legal, ethical, social and cultural issues that arise during their development and deployment. A multidisciplinary effort was subsequently commissioned and over 40 experts recruited to contribute at various stages of development. In accordance with the recommendations, the group included many members who possessed a broad knowledge in their topic areas but were not involved directly in research on GMMs. A draft guidance framework was produced and opened for public comment in late 2012. Responses to public comment have been incorporated into this current version.

Because of the breadth of different genetic approaches that are under consideration and conditions under which they might be used, it is not possible to provide an exact formula for evaluation of all GMM technologies. It will be necessary to determine the specific needs on a case-by-case basis. Thus, the guidance framework presented here does not offer precise instructions for testing GMMs, but rather aims to support informed and thoughtful process development. Efficacy and safety testing standards are proposed that are complementary to those used for trials of other new public health tools, including drugs, vaccines and insecticides, drawing also from relevant experience in agriculture and biocontrol. The guidance framework examines the fundamental considerations for addressing public engagement and transparency needs in research on GMMs, taking into account lessons learned from previous introductions of new technologies in the fields of health and agriculture. Finally, while it reviews existing regulatory requirements and guidance that are either directly pertinent to research on GMMs or may provide precedents for establishing the appropriate level of oversight, it is understood that such precedents will continue to be expanded and refined as research on modified mosquitoes proceeds. This *Guidance Framework for Testing of Genetically Modified Mosquitoes* is intended to foster quality and consistency in the processes for testing and regulating new genetic technologies. It is hoped that it will contribute to comparability of results and credibility of conclusions in addressing the requirements for decision-making by countries interested in the potential use of these technologies as public health tools for control of vector-borne diseases.

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Key messages

1. Despite ongoing control efforts, diseases transmitted by mosquitoes, such as malaria and dengue, continue to pose an enormous global health burden. Multinational public health organizations have called for the eradication of malaria and of the major mosquito vector of dengue. There is broad recognition of the need for improved tools to combat these diseases, including tools for vector control.

2. Currently available methods to control mosquito vectors of malaria and dengue are based on the use of insecticides and elimination of mosquito larval breeding sites. In considering the potential of new technologies to address the unmet needs of mosquito control, it is necessary to evaluate their risks and benefits in the context of the current situation. Thus, the risk incurred by testing new and unproven strategies should be weighed against the risks to human health and the environment posed by maintaining the status quo, which includes both ongoing disease and exposure to broad-spectrum insecticides, and of the changing status of factors affecting mosquito abundance, such as land use, urbanization and climate.

3. GMMs have been proposed as a possible new tool to reduce transmission of malaria and dengue. This *Guidance Framework* is intended to foster quality and consistency of procedures for testing of GMMs, which will contribute to comparability of results and credibility of conclusions in addressing the needs for decision-making by those considering the use of GMMs as public health tools to control mosquito-borne diseases. The *Guidance Framework* should be useful to readers interested in:

    - GMM technologies and applications that currently are being contemplated;
    - safety, efficacy, regulatory and social/ethical issues involved in taking GMMs from the laboratory to field testing;
    - precedents that exist for how these issues have been dealt with to date;
    - existing regulatory frameworks and international agreements that are relevant to GMM testing and eventual implementation.

4. GMM technologies currently under development are aimed at either reducing the size of the mosquito vector population to an extent that will significantly reduce pathogen transmission ("population suppression") or at replacing the current population with mosquitoes that have been made less capable of transmitting a particular pathogen ("population replacement").

5. These technologies can be further defined according to how long the GMMs are intended to persist in the environment following release. The persistence of the GMM effect will depend upon the transgene components and their behaviour.

6. With "self-limiting" approaches, the genetic modification is designed to decline in frequency within the mosquito population over time until it disappears. In some cases, the GMMs are meant to be sterile and thus unable to pass the genetic modification on to future generations through mating. In other cases, the GMMs are meant to mate and introduce the effect briefly

into the local mosquito population, but it is expected that crossing with local mosquitoes over a number of generations will reduce the modification until it is lost. Thus, the protective effect of self-limiting approaches can only be maintained by periodic re-releases of GMMs, and how often these releases must be performed will depend upon the type of genetic modification. From a RA perspective, these releases can be readily halted and this should decrease the possibility of producing undesirable changes in the environment. However, the need for frequent reintroductions is associated with ongoing costs of production and delivery.

7. With "self-sustaining" approaches, the genetic modification is intended to be spread into the local mosquito population and to persist indefinitely. These approaches have the potential to provide highly durable and cost-effective protection against pathogen transmission, but any unforeseen effects may be more difficult to reverse than would be the case for self-limiting approaches.

8. GMM technologies offer several theoretical advantages over conventional vector control strategies. They may reach mosquito populations and mosquito larval breeding sites that have traditionally been the hardest and most expensive to access by exploiting the natural behaviour of mosquitoes to mate and seek sites for egg laying. For example, GMMs would be well suited to urban settings, where current control measures are largely ineffective due to the wide availability of cryptic mosquito larval breeding sites. Additionally, GMMs may reach outdoor and day-biting mosquitoes that often escape control methods such as bed nets and indoor insecticide spraying. The modification could be made highly specific for the target mosquito species, which would avoid ecological and environmental hazards associated with commonly used broad-spectrum insecticides. GMMs could provide continuous protection in situations where other disease control methods have been interrupted, and prevent the reintroduction of the pathogen after successful elimination efforts. It is important also to note that GMM technologies could be used in ways that are compatible with other disease control methods and could be incorporated into integrated vector management programmes.

9. Theoretical disadvantages also have been raised for GMMs, including several unknowns related to possible ecosystem interactions. Because of the breadth of different genetic approaches that are under consideration as well as conditions under which they might be used, it is not possible to provide a universal formula for evaluation of GMM technologies. As with other public health technologies, case-specific testing will be required to understand the advantages and disadvantages of a particular GMM approach, keeping in mind both the potential benefits as well as risks. This can begin prior to field-testing as particular GMM approaches are developed, building on principles already described for existing technologies.

10. A phased testing pathway is recommended for GMMs, analogous to the development pathway for other new public health tools, with systematic assessment of safety and efficacy at each step. New GMM technologies would first move from the laboratory (Phase 1) to testing under confined conditions that provide a more natural setting but still limit release into the environment (Phase 2). Phase 2 may involve testing under physical confinement, as in a large cage equipped to simulate a disease-endemic setting, or under ecological confinement, as under geographic, spatial or climatic isolation. RA and prior experience with the technology will inform the plan for confined testing; it is recognized that regulatory requirements for physical and

ecological confinement will differ because of the different levels of environmental exposure. Following confined testing, GMMs may proceed to a series of staged open release trials in Phase 3, designed to measure performance under different conditions and to assess the ability of GMMs to reduce infection and/or disease in human populations. Based on results from Phase 3, a decision may be made to deploy GMMs as a public health intervention (Phase 4). Phase 4 would be accompanied by a plan for long-term monitoring of safety and efficacy.

11. The transition from one phase to the next will be subject to "go/no-go" decision criteria, including efficacy and safety endpoints, regulatory and ethical approvals, and social acceptance. Testing would not proceed if either the responsible regulatory authority or the developer makes a "no-go" decision or places a trial on hold in order to collect more information. Community acceptance would be a critical determinant in deciding whether testing could move forward in a particular location.

12. The critical path for GMM development will include not only proof of efficacy, but also proof of acceptability and deliverability. Risk analysis, community and other stakeholder engagement, and regulatory approval all contribute to proof of acceptability. Cost-effectiveness of the technology vs. other available disease control methods also may influence acceptability. Deliverability will require consideration of an operating model with appropriate prospects for financing to support deployment and subsequent monitoring, sufficient technical and production capacity, quality control processes, methods for management and mitigation in the case of adverse effects, as well as commitment to ongoing stakeholder engagement.

Efficacy evaluation

13. GMMs must be effective in reducing transmission of the targeted pathogen(s) and not detrimental to the environment and human health if they are used as public health intervention tools. Demonstration of efficacy will be a critical determinant for decision-making about deployment.

14. The efficacy of GMMs may be measured by both entomological and epidemiological endpoints. The entomological endpoint is a reduction in the risk of disease transmission as measured by specific mosquito population characteristics. The epidemiological endpoint is a reduction in the incidence of infection or disease in human populations. Whereas entomological endpoints may be relevant through all phases of testing, epidemiological endpoints will probably only become significant as research progresses to larger trials under Phase 3.

15. The most direct measure of an entomological endpoint is a reduction in the estimated transmission intensity, which is called the entomological inoculation rate (EIR). Because measuring EIR reductions is difficult or impossible during Phase 1 and Phase 2, it will be necessary to infer reductions in EIR by surrogate vector indicators that would contribute to the EIR, such as vector population size, transgene frequency, GMM fitness, or pathogen replication within the vector.

16. A potentially powerful design for determining efficacy of GMM applications is the cluster randomized trial. Such trials must be designed to allow measurable reductions in an endpoint such as infection incidence. Careful site selection is necessary to increase the likelihood of

detecting significant results. The influence of seasonal and inter-annual variations and spatial heterogeneity in incidence on trial design must be considered. "Go" and "no-go" criteria for moving forward should be determined. Independent monitoring of trials is recommended.

17. GMMs will most likely be applied in the context of conventional control measures. Thus, the effect of other ongoing control measures on the outcomes of the GMM trials must be considered in the trial design. The efficiency of GMMs relative to conventional control will in part determine their utility.

Biosafety

18. Risk is the likelihood that harm will occur from a particular action. The level of risk is estimated as the product of the expected probability that a harmful event will occur and the expected consequences, or impact, of the event.

19. RA is a methodological approach to systematically define the level of risk. Risk management (RM) encompasses strategies developed to avoid and reduce risk to acceptable levels. Risk analysis encompasses RA and RM, as well as risk awareness and risk communication. Risk analysis should articulate and inform the concerns on which to focus and the acceptability of risks, and convey the results of these processes to the public and to decision-makers.

20. The core functions of risk analysis are assessment and management. RA should determine: the planned actions and potential routes of exposure for defined hazards, how these can be measured and the limits of concern; a characterization of events leading to potential negative impacts of the GMMs; the anticipated level of exposure to these events leading to quantification of the likelihood and consequences of their effect on target organisms, non-target organisms (NTOs) and human health; and the levels of uncertainty associated with the potential events, levels of exposure, and their consequences. RM should identify and evaluate proportionate measures that are needed to mitigate any harm or uncertainty and demonstrate how both standard and responsive measures would make the identified risks acceptable to regulators. Additional risk communication may be needed to determine that RM is also acceptable to a wider community.

21. The evaluation of risk should be set against the benefits of GMMs for improving human health on a case-by-case basis. Cost-benefit or cost-effectiveness analyses can provide the framework under which the appropriate (economic, health, social) returns of a GMM-release programme may be quantified, and provide a context for decision-making about the level of acceptable risk. RA of novel technologies should be set against the risk of relevant alternatives, such as the risk of no action or the risk of conventional control methods. For example, "causes more harm" than current practice is a reasonable comparator for RA of GMM-based vector control systems.

22. On evaluation, risk in some cases may be judged as negligible, as when the probability a harmful event will occur is determined to be very low or the consequences of an event occurring would be minimal. Moreover, in many cases, despite potentially harmful events being identified, the practical level of risk to which the public is exposed can be reduced to acceptable levels by effective management. The identification of potential hazards does not in itself indicate an unacceptable risk.

23. Biosafety considerations in Phase 1 testing of GMMs should include:

- how appropriate comparators will be chosen, what appropriate comparisons should be made, and what endpoints will be used for these comparisons of risk;
- stability and effectiveness of the transgene at the population-level and the consequences of incomplete or partial transgene function;
- the phenotype of GMMs with multiple transgenes, rather than the effect of individual genes;
- the methodology for and impact of sex separation, if appropriate to the GMM technology being assessed;
- how GMMs will be discriminated within a wild population after release, how the maintenance of gene integrity will be monitored, and how trial endpoints will be determined;
- the type, strength and function of the appropriate ecological processes affecting the GMM population;
- appropriate ecological and biological comparisons for NTOs.

24. Additional biosafety considerations in Phase 2 testing should include:

- determination of the need for physically confined testing prior to ecologically confined testing;
- appropriate site selection criteria for confined trials, bearing in mind the spatial location, timing and duration of ecologically confined field trials;
- spatial extent of the trial, including potential risks in areas outside the designated trial site(s);
- development of detailed standard operating procedures (SOPs) to ensure that rearing, release and monitoring are carried out consistent with the relevant assumptions made in RA, with clear lines of responsibility and reporting, and RM strategies for field trials;
- potential for unanticipated effects on disease burden;
- non-target species assessments, if appropriate, for confined field trials.

25. Additional biosafety considerations in Phase 3 testing should include:

- characterization of local target mosquito ecology as required to set appropriate trial endpoints, including impact on human health and the wider environment;
- methods for evaluating GMM success through population-level assessments;
- appropriate RM plans for any potential resistance to the genetic modification, designating the lines of responsibility for managing this risk;
- proportionate assessment and management of non-target and off-target effects and the likely risk of transgenic gene flow;
- proportionate assessment and management of risks associated with the mass production of mosquitoes.

26. If and when a decision is made to deploy GMMs broadly as a public health tool, there may be a need for post-implementation quality control and surveillance to monitor for effectiveness and development of specific risks identified by post-release assessment. Biosafety considerations in Phase 4 should include:

- methods available for ongoing monitoring of the epidemiological impact of GMMs on human health;

- methods available for ongoing monitoring of safety for the environment and human health (in a manner analogous to pharmacovigilance, the monitoring applied to medicines after introduction to market);
- available mitigation methods in the case that a negative effect is observed;
- risk implications and management of the movement of GMMs across borders.

27. Independent ongoing safety review during testing is recommended, covering relevant aspects of environmental monitoring and human health. This may be accomplished through existing institutional or national level biosafety committees or through the establishment of new review bodies focused on GMM activities. The strengthening of biosafety oversight capabilities within disease endemic countries should be encouraged. National biosafety laws and regulations developed primarily to regulate genetically modified (GM) plants may need to be reinterpreted for GMM, or additional guidance provided.

Ethics and public engagement

28. In the design of GMM trials, a key set of questions relates to the ethical implications, including the nature and scope of the obligation to respect host communities and what type of protections should be provided to them. Respect for communities should be understood as an overarching ethical goal within GMM trials.

29. Although activities of ethical reflection and engagement often overlap with those of regulatory compliance, ethical issues and responsibilities are generally broader than just those activities specifically mandated by administrative law or organizational policies. It should not be assumed that regulatory compliance implies that ethical and community engagement responsibilities have been addressed adequately.

30. Democratic governance of technology requires that proposals on issues such as the testing of GMMs be discussed and debated openly in a manner that receives the attention of scientists and decision-makers, and in a way that ensures that stakeholders' voices can be heard.

31. The ethics and engagement component of a GMM research programme will take place at multiple levels, three of which are mentioned below.

    - **Within the project team.** Team members and their advisers should articulate the value and social purpose of the research, engage in ongoing and structured ethical reflection (including consideration of dissenting opinions and legitimate public concerns), document publicly the ethics and engagement activities that have been done, and evaluate the performance of these activities. All of these efforts should contribute to further development and refinement of plans and methods.
    - **With the host community.** Researchers have ethical responsibilities to people living within a trial site. For that subset of individuals classified as "human research subjects" according to standard regulatory criteria, informed consent obligations will apply. However, there may be many individuals living within a trial site who are not, in a traditional sense, subjects of the research at hand, but who nonetheless may be affected by the conduct of research. Community engagement addresses ethical obligations to these people, including undertaking procedures that would be expected to identify them, advising them that they may have

interests at stake, finding out what concerns they may have, responding to those concerns, and reaching some form of agreement about whether the trial should proceed.

- **With third parties.** Individuals *not* immediately associated with the trial site such as public health or international development organizations, other scientists, members of CSOs, the press, and the general public, will take an interest in the conduct and outcome of the research. The ethical obligation to third parties is not to seek them out proactively to ensure awareness of the research, but to consider and respond to their expressed concerns and interests in a respectful manner. GMM projects should incorporate a communications/public engagement strategy that includes education about the goal and methods, but also provides opportunities for follow-up discussion.

32. Ethics and engagement activities should be considered before Phase 1 proof-of-concept work has been completed. Adequate plans for communication and engagement should be put in place before the earliest stages of field testing begin. Community engagement activities should begin during the collection of baseline entomological data, in order to avoid the possibility of misunderstandings and miscommunications that could undermine respect for the host community and jeopardize future research. Plans also should include initiating interactions with policy-makers to explain research goals and develop an open dialogue.

33. Community engagement and authorization activities will be necessary in Phase 2 of the GMM testing pathway. Before proceeding to confined release trials, plans should be in place for responding to ethical obligations to individuals being asked to participate as human research subjects and/or to communities being asked to host trials. Communications should explain that trials are research activities intended to test a new technology, a protective effect is not assured, and the community must continue to employ other available methods to protect themselves from disease transmission.

34. Community engagement and authorization activities will expand in Phase 3, and human subjects issues will become more prominent in trials undertaken to determine the epidemiological impact of GMMs.

35. In Phase 4, ethical responsibilities to those who are affected by the technology are increasingly likely to converge with established processes. Deployment of GMMs will be a public health initiative and will take place in the context of existing legal, regulatory and political institutions. However, the need for public engagement activities is likely to continue.

36. It will be important for members of the scientific team to be involved in ethics and engagement activities. However, many aspects of these activities will also require the specialized skills of social scientists and communications experts. Adequate funding for these activities will be imperative for the successful accomplishment of the research objectives.

37. A need can be anticipated for training of project scientists about research ethics, and of institutional or national ethics review committees in the specialized issues associated with vector biology research.

Regulatory frameworks

38. Regulation is an enabling process that ensures that safety and efficacy are consistent with social values. Regulation of GMMs may be encountered early in the research process and throughout development and implementation. Regulation can be expected at institutional, state, provincial and national levels, all of which may have to be addressed concurrently.

39. Each country has its own sovereign regulatory process, but overarching international agreements or treaties also may be relevant. Early investigation of the regulatory processes in a given country and open communication with the national officials, risk assessors, and decision-makers are imperative in order to understand the requirements relevant to GMMs.

40. Early interaction with regulators will serve to identify the appropriate regulatory pathway for GMMs, and proactive communications will help to build understanding within regulatory agencies about the GMM technology, as well as the goals and methodologies of the project. There may be a need to strengthen familiarity with entomology research methods and/or biosafety procedures, and this should be planned for accordingly.

41. The Cartagena Protocol on Biosafety (CPB) is accepted by almost all developing countries and is anticipated to be an important influence on GMM regulatory processes and RAs. It will be essential to work with regulators to ensure understanding of the differences between GMM and GM plants or crops, including the fact that human health benefits are relevant as part of the regulatory decision-making process for GMMs. Limited resources available to GMM developers, especially where products are intended primarily to serve the public health needs of developing countries, make it important for authorities to exercise discretion in imposing regulatory requirements, taking into account scientific rationale and relative risks.

42. Regulation of GMMs may present unanticipated costs and potential delays that must be recognized as early as possible. Plans for dealing with such contingencies should be put in place and suitably resourced.

43. Informed public involvement and consent in the GMM regulatory decision process is a necessity if implementation is to occur without adverse public reaction. Regulatory processes often include formal public consultation opportunities.

44. While there is currently no standardized procedure for addressing potential transboundary movement of GMMs that are self-sustaining or with gene drive, some precedent is provided by prior introductions of classical biological control agents in agriculture. A regional notification and agreement process may be advisable for planned introductions capable of autonomous international movement beyond the scope of provisions in the Cartagena Protocol and may best involve a multilateral organization in a coordinating capacity.

1. Introduction

Summary: The need for better methods to combat mosquito-borne diseases is widely recognized. Recent research offers the possibility that genetically modified mosquitoes (GMMs) could be used to prevent pathogen transmission. GMMs provide several theoretical advantages that may make them attractive for vector control, such as specificity and the ability to function in areas that are difficult to reach with conventional control methods. Different GMM technologies under consideration include those aimed at reducing the number of mosquito vectors in a given region (population suppression) or rendering the local mosquitoes unable to transmit a pathogen (population replacement). Both types of technologies can be designed so that GMMs persist for only a brief period of time (self-limiting) or so that the modification is passed on through local wild mosquitoes and persists indefinitely within the local mosquito population (self-sustaining).

Ongoing releases of self-limiting GMMs will be required to maintain effectiveness. Self-limiting approaches may be attractive from an environmental safety perspective since they are not expected to persist in the environment or to spread far beyond the release site. However, self-sustaining approaches ultimately could provide more durable and cost-effective public health solutions. A phased testing pathway is recommended, in which new GMM strategies move from the laboratory, to testing in more natural environments under confined conditions, and finally to open release trials, with each transition dependent upon satisfactory demonstration of efficacy and safety. When GMM are incorporated into national or regional vector control programmes, the need for ongoing case-specific monitoring of effectiveness and safety should be considered to ensure acceptable quality and performance standards and to inform any necessary management responses.

Current mosquito control efforts rely heavily on chemical methods including insecticide-treated bed nets, indoor residual spraying with insecticides, outdoor insecticide fogging, and application of chemical larvicides, or management of standing water for mosquito larval breeding sites. Despite diligent application of available control strategies, including improvements and expanded use of bed nets, mosquito-borne diseases such as dengue (WHO, 2012),[3] and malaria (Murray et al., 2012; WHO, 2013) continue to pose major global health challenges. WHO experts have stated that, "global eradication of malaria cannot be expected with existing tools" due to the difficulties of interrupting transmission in sites with ongoing high vectorial capacities (Mendis et al., 2009). Malaria mapping and modelling studies support this conclusion (Hay et al., 2009, Griffin et al., 2010). Similarly, a WHO Special Programme for Research and Training in Tropical Diseases (WHO-TDR)-sponsored dengue scientific working group acknowledged that, "we are collectively failing to meet the threat posed by dengue as the disease spreads unabated and almost 40% of the world's population now live at risk of contracting it" (Farrar et al., 2007). Re-emergence of dengue over the last two decades is exacting an increasing public health and economic toll (Shepard et al., 2011, Shepard et al., 2013). The disease is now recognized as one of the most common reasons for hospital admission in the Americas and Asia during the rainy seasons (Whitehorn & Farrar, 2010). WHO has acknowledged that, "innovative vector control tools are badly needed," and in particular that, "methods that improve the ability to deliver persistent treatments more rapidly and efficiently into large urban

[3] Dengue and severe dengue: http://www.who.int/mediacentre/factsheets/fs117/en/, accessed 25 May 2014.

communities in a sustained way are urgently needed" (WHO, 2012). Limitations of current vector control methods include: inability to reach mosquito larval breeding sites and adult resting sites; evolution of resistance to chemical agents; compliance and infrastructure issues; concern about the impact on the environment and/or toxicity to humans; and, importantly, cost. The ongoing costs of vector control are substantial,[4] and maintaining the high levels of donor and national government support necessary to achieve high coverage of control measures over long periods of time has historically proven daunting (Mills, Lubell & Hanson, 2008; Leach-Kemon et al., 2012). Thus, for both operational and economic reasons, there is a recognized need for new, sustainable, and cost-effective vector control tools.

Intensive interest arose in the late 1980s for the application of modern genetic engineering technology to arthropod vectors as a useful approach for limiting transmission of human pathogens (Beaty et al., 2009). Subsequent research has focused in large part on two high impact mosquito species, *Anopheles gambiae* and *Aedes aegypti*, which serve as major vectors for malaria and dengue, respectively.

Substantial progress has been made on challenges such as sequencing the genomes of these two important vector species, achieving stable germline transformation, identifying sex-, tissue- and stage-specific DNA control elements, identifying genes involved in susceptibility or resistance to infection/insecticides, and developing models for methods to spread heritable modifications into native mosquito populations within an epidemiologically relevant timeframe as needed to achieve disease control. The initial technical objective, germline transformation, has been accomplished in all major mosquito genera (Allen et al., 2001; Catteruccia et al., 2000; Jasinskiene et al., 1998) and can be considered routine for several species. Beyond similar preliminary achievements, effector genes have been developed that accomplish proof of principle for either refractoriness or sterility. Examples include: 1) mosquitoes refractory to malaria parasites (Ito et al., 2002; Corby-Harris et al., 2010, Isaacs et al., 2011; Isaacs et al., 2012) and dengue virus (Travanty et al., 2004; Franz et al., 2006); and, 2) mosquitoes that are sterile (Windbichler, Papathanos & Crisanti, 2008) or that function in a manner to limit reproductive potential (Fu et al., 2010; Galizi et al., 2014; Phuc et al., 2007; Thomas et al., 2000). Additional methods have been proposed or demonstrated that await development in transgenic mosquitoes (e.g. Marshall et al., 2010; Papathanos et al., 2009; Schliekelman & Gould, 2000). Efforts can also be envisioned to develop additional effectors to reduce life span or alter behaviours in a beneficial way.

Although much work remains to be done, it is now possible to envision a pathway towards the realization of the successful implementation of genetic technologies for the control of mosquito-borne diseases. A multidisciplinary effort will be required, encompassing not only additional scientific advances, but also complementary planning for ethically and environmentally responsible testing as well as for reliable, cost-effective and socially acceptable deployment. Consequently, the technical consultation on GMMs organized in May 2009 by WHO-TDR and the Foundation for the National Institutes of Health (FNIH) recommended that a guidance framework be developed for assessing safety and efficacy and addressing regulatory and ethical, social and cultural issues during

[4] Global Malaria Action Plan, Table II.4: http://www.rollbackmalaria.org/gmap/2-5.html, accessed 25 May 2014.

the development and testing of GMMs (WHO, 2009). The framework presented here is intended to provide a basis for conduct of trials according to best practices that will contribute to comparability of results and credibility of conclusions. This should facilitate decision-making by countries regarding the potential testing and use of GMMs as public health tools for prevention and control of malaria, dengue and other mosquito-borne diseases.

1.1 GMM technologies

Currently contemplated GMM technologies are designed to have the following two major types of effect.

- **Population suppression** – strategies that target vector "demography" with the intent to reduce (suppress) the size of the mosquito population such that it would not be able to sustain pathogen transmission. These include methods to reduce the overall numbers of female mosquitoes (with or without a concomitant direct effect on males), which will result in decreased reproduction. Examples of how this could be accomplished include biasing against the development of female progeny (sex-ratio distortion), reducing female fertility, or introducing a mechanism that incapacitates or kills young female mosquitoes. This category also includes methods to shorten the lifespan of female mosquitoes, thus decreasing the length of time available both to transmit a pathogen from one person to the next and to reproduce.
- **Population replacement** – strategies that target vector competence with the intent to reduce the inherent ability of individual mosquitoes to transmit a given pathogen. This involves the introduction of engineered DNA and/or the manipulation of endogenous genes so as to inhibit pathogen replication within the mosquitoes, making them refractory to transmission of particular viruses or parasites. Upon release into the environment, these refractory GMMs will be expected to introduce, through mating, the change into the local mosquito population, "replacing" their inherent ability to spread the targeted pathogen with a reduced or eliminated transmission capability.

These strategies can be further categorized according to the ability of GMMs to persist following release (Table 1.1; Alphey, 2014). This will depend largely on a combination of two characteristics. The first is "fitness cost" (a decrease in the mosquito's ability to survive and reproduce as a result of the genetic modification) and the second is "drive" (a mechanism to increase the frequency of effector genes in a population at a rate faster than would be expected through normal Mendelian inheritance). The following two general approaches are being pursued.

- ***Self-limiting*** *– approaches in which the GMMs are unable to pass the modification on indefinitely through subsequent generations.* Self-limiting approaches are designed to impose a significant fitness cost, which will cause the GMMs to decline in frequency over time until they disappear within the local population unless they are maintained by periodic new releases. In general, the greater the fitness penalty, the shorter the time period over which the GMMs would be expected to maintain their effectiveness. Indeed, a subset of the self-limiting approach is comprised of GMMs that limit the number of viable adult progeny produced from mating and hence the amount of genetic material passed to future

generations. In this case, the genetic modification may aim for "sterility" (the GMMs do not reproduce) or late-acting lethality (the GMMs reproduce but most of their progeny do not survive to adulthood). Other self-limiting approaches impose a less severe fitness cost, and therefore the modification will disappear more gradually from a population when releases stop. Some of these are designed to have a transient gene drive system that breaks down over time, at which point harmful effects on fitness predominate and the modification is expected to disappear from the population without recurrent releases. Thus, with self-limiting approaches, the combined effect of the fitness cost, which works against persistence, and drive, which promotes persistence, will dictate how long the GMMs will remain effective in the field and how often additional releases will be required.

A spectrum of different self-limiting approaches is under development. Some are being constructed to function similarly to the sterile insect technique (SIT) that has been used successfully against pest insects affecting livestock and crops (Lindquist et al., 1992; Dyck, Hendrichs & Robinson, 2005). In this case, few, if any, viable offspring are expected to result from the mating of GMMs with native mosquitoes. The reproductive potential of the local population, therefore, is expected to decrease, resulting in population suppression. Such approaches will require frequent inundative releases of GMMs to maintain effectiveness. With self-limiting approaches at the other end of the spectrum, i.e. those that impose a lower fitness cost and incorporate weak drive, GMMs from an initial release are expected to mate productively with local mosquitoes and introduce the desired effect into the population. However, the modification will gradually be diluted over a number of generations of crossing with native mosquitoes until it is lost. Less frequent releases, involving lower numbers of GMMs, would be required to maintain the effectiveness of this type of self-limiting approach.

Computer simulations support the potential for self-limiting approaches to substantially reduce vector-borne diseases (e.g. Atkinson et al., 2007, Legros et al., 2012). Moreover, it has been argued by some that release of self-limiting constructs should constitute the early stages of field testing in order to gain experience with GMM technology under circumstances where its effects could be withdrawn by halting releases (Benedict & Robinson, 2003).

- **Self-sustaining** – approaches in which heritable modifications are intended to spread indefinitely through the target population. Self-sustaining approaches must be able to spread the effector mechanism into native mosquito populations within an epidemiologically relevant timeframe. Thus, they require a strong drive mechanism capable of overcoming any fitness costs and increasing rapidly the frequency of the effector gene(s) from low initial levels to fixation, or near fixation. Once established, self-sustaining approaches are intended to be relatively stable and to require smaller and infrequent inoculative releases to maintain effectiveness. In the case of population replacement, the modification may become fixed permanently within the local population. With self-sustaining population suppression strategies, the modification may spread until the local vector population is greatly reduced or

eventually eliminated. Computer simulations support the potential for self-sustaining approaches to provide complete elimination of the disease pathogen in some circumstances, potentially replacing existing control methods (e.g. Deredec et al., 2012).

Table 1.1 GMM technologies currently under development

Strategy	Approach	
	Self-limiting	Self-sustaining
Population suppression	- Modification reduces the number of progeny - Possesses either no gene drive or weak drive that will pass the modification through only a limited number of generations - Not intended to persist in the absence of continued releases	- Modification reduces the number of progeny - Possesses strong gene drive - Intended to spread the modification indefinitely or until the mosquito population is eliminated
Population replacement	- Modification limits pathogen replication, thereby reducing transmission - Possesses weak gene drive that will pass the modification through only a limited number of generations - Intended to persist only until diluted out of the population	- Modification limits pathogen replication, thereby reducing transmission - Possesses strong gene drive - Intended to spread the modification through the population indefinitely

1.2 Characteristics of GMMs

GMM technologies offer certain potentially favourable design characteristics as new vector control tools.

- They could provide area-wide protection that is accessible to everyone, regardless of their socioeconomic level, and they do not require people to change their behaviour in order to be effective.
- They would not require application of a chemical that must come into direct physical contact with the mosquito to be effective.
- They could reach mosquito populations and their larval breeding sites that have been traditionally the hardest and most expensive to reach using conventional vector control strategies by exploiting the natural seeking behaviour of the mosquitoes to find mates and oviposition sites. This would include outdoor and/or day-biting vectors that escape control by bed nets and indoor spraying but may play an important role in transmission.
- A high level of specificity and stability would reduce ecological, environmental and human health hazards associated with currently available broad spectrum insecticides.

- They would be well suited to application in urban environments where current control measures largely have proven inadequate.
- Technologies aimed at population suppression could reduce transmission of all pathogens transmitted by the same vector mosquito. For example, suppression of *Aedes aegypti* vectors could reduce transmission of dengue, yellow fever and chikungunya viruses.

Self-sustaining approaches have additional envisioned characteristics that would be useful in disease elimination or eradication efforts.

- Limited need for reapplication would minimize the requirement for ongoing mass production and delivery, which should make their use relatively inexpensive.
- Durability of activity should maintain effectiveness even in situations where other disease control methods must be temporarily suspended, as, for example, due to adverse weather conditions or civil unrest.
- Population replacement technologies would reduce or eliminate the pathogen, rather than a particular mosquito vector. By not leaving an empty ecological niche, their effects should not be limited by the potential for invasion of the treated area by other competent vectors.
- Some of the technologies could affect more than one local vector species if cross-mating occurs even at low levels, thus having the potential to reduce disease in regions where it is transmitted by related species.

Theoretical disadvantages of GMMs also have been proposed. These include possible ecosystem effects. An example is the complexity of applying a species-specific technology in situations where disease is spread by multiple vectors and the possibility that removal of the current disease vector may allow a new vector to become established. Other potential issues are the development of resistance over time, either on the part of the mosquito or the pathogen, and the loss of immunity by people in treated areas over time; however, these possibilities also are shared by other control methods such as insecticides and drugs. Such possible hazards must be taken into consideration in risk assessment (RA) (Section 3. Biosafety).

1.3 Potential utility of GMMs

GMMs are primarily being developed for use within disease endemic or epidemic situations as part of an area-wide control programme to reduce the rate of pathogen transmission. GMMs are likely to be used as part of an integrated approach, in conjunction with other disease control methods. GMMs are compatible with use of drugs and vaccines, as well as common vector control methods such as source reduction. Importantly, GMM-mediated methods to reduce the force of disease transmission by reducing the number of infectious bites could improve the protective potential of new vaccines. For example, modelling suggests that a pre-erythrocytic malaria vaccine would be much more effective in low transmission settings than in high transmission settings (Penny et al., 2008). Likewise, concurrent use of a vaccine would reduce the possibility that prolonged reduction in pathogen exposure due to effective transmission control might result in loss of immunity within the human population (Ghani et al., 2009).

Because they would not require a high level of individual participation, GMMs may not be as susceptible to the lack of compliance that is sometimes seen with conventional control programmes after disease rates fall and the perceived threat is low. Ongoing area-wide protection provided by GMMs, especially those that are self-sustaining, could prevent the reintroduction of the pathogen into the population (for example, by immigration of infected persons or mosquitoes) after successful regional elimination efforts. This may provide a valuable tool for disease eradication.

Certain GMM technologies could also be useful as a preventative measure in regions where disease is not yet occurring. For example, where exotic mosquito species may be introduced, GMMs could help to prevent their establishment. This is analogous to current utilization of SIT to prevent Mediterranean fruit fly infestation in otherwise pest-free areas.

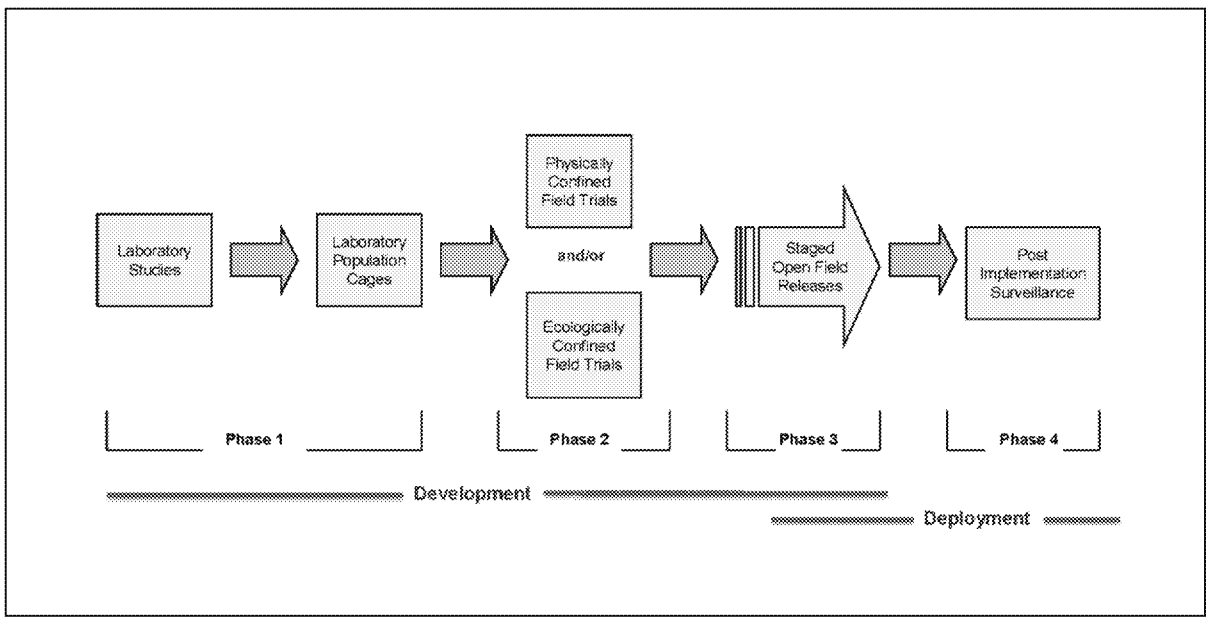
1.4 GMM testing pathway

A series of workshops held in London and Atlanta in 2001 (Alphey et al., 2002), Wageningen in 2002,[5] and Nairobi in 2004,[6] began a process to discuss requirements related to the testing and implementation of genetically modified (GM) vectors. The concept of phased testing was widely advocated. The recommendation to develop a phased testing pathway was reiterated at a technical consultation, held in Geneva in May 2009, which focused on practical and technical issues associated with moving new GMM technologies from the laboratory to field testing (WHO, 2009).

In accordance with these earlier recommendations, a stepwise testing process as illustrated in Figure 1.1 is proposed in this guidance framework. Subsequent sections expand upon specific considerations related to efficacy testing, safety testing, ethical, social and cultural issues, and regulatory decisions to be addressed at each phase.

[5] Ecological aspects for application of genetically modified mosquitoes: http://library.wur.nl/frontis/malaria/index.html, accessed 25 May 2014.
[6] Bridging laboratory and field research for genetic control of disease vectors: http://library.wur.nl/frontis/disease_vectors/index.html, accessed 25 May 2014.

Figure 1.1 Phased testing pathway for GMMs



For simplicity, the illustration describes a unidirectional pathway. In practice, however, repetitions of some segment(s) of the pathway may be required in order to improve the technology and refine the procedures until the requirements for moving to the next phase are met.

Phase 1 is anticipated to begin with small-scale laboratory studies for efficacy and safety testing, followed by testing in larger population cages in a laboratory setting conducted under appropriate containment facilities and procedures.[7] Laboratory testing under highly controlled conditions will allow preliminary assessment of whether the GMMs demonstrate the desired biological and functional characteristics, with an eye toward future efficacy and safety.

For those GMMs showing promise in Phase 1, **Phase 2** initiates confined testing in a more natural setting but under conditions that will limit release into the environment. Small trials in Phase 2 may involve testing under physical confinement (sometimes termed "containment") within a large cage that simulates the disease-endemic setting while minimizing the possibility for escape. In the early stages of testing of mosquitoes incorporating gene drive, experts have advocated testing under physical confinement, such as within a greenhouse or screen-house type facility (Alphey et al., 2002; Scott et al., 2002, Benedict et al., 2008). Phase 2 testing also may involve small-scale ecologically confined field release. Ecological confinement entails geographic/spatial and/or climatic isolation intended to limit the spread of GMMs into the environment. The decision about requirements for one or both components of Phase 2 testing will be made by the national regulatory authority and will probably depend on the nature of the GMM technology, prior knowledge of its effects in other

[7] For example, arthropod containment levels: http://www.liebertonline.com/doi/pdfplus/10.1089/153036603322163475, accessed 25 May 2014; or Australian Government (2006).

environments and other factors that are taken into account in the process of RA (Section 3. Biosafety). A situation in which a physically confined trial might not be deemed necessary might arise, for example, when a technology has already been tested and found to be safe in another venue. It should be noted, however, that the regulatory requirements for physically vs. ecologically confined trials are expected to be different, since an ecologically confined trial involves intentional, although limited, release into the environment. Phase 2 trials will continue the assessment of biological and functional activity of GMMs, including their effect on local/wild-type mosquitoes, but because of their limited scale will only rarely provide information on the disease impact of the technology. Moving on to initiation of larger GMM trials in the environment and in disease-endemic countries will require thoughtful consideration and the application of relevant ethical and regulatory practices (Section 4. Ethics and public engagement; and Section 5. Regulatory frameworks).

Contingent upon satisfactory results of confined testing in Phase 2, the GMM technology may proceed to staged open release trials under **Phase 3**. It is likely that this will involve a series of sequential trials of increasing size, duration and complexity, to be conducted at a single site or multiple sites. These trials may be designed to assess performance under various conditions, such as different levels of pathogen transmission, seasonal variations in mosquito density, or presence of other disease vectors in the region. While measurement of entomological parameters is likely to remain the focus of early Phase 3 trials, later trials in this phase may include measurement of the impact of GMMs on infection and/or disease in human populations. Trials to show epidemiological impact must be designed accordingly, with considerable thought on the needs for achieving a statistically meaningful result. Although still focused on intense examination of the function and efficacy of GMMs, Phase 3 trials effectively institute a limited deployment of the technology; this will especially be the case for self-sustaining approaches that are anticipated to persist.

Approval for moving forward to each consecutive phase of testing (phases 1–3) will be the responsibility of the relevant national regulatory authority. The identity of this authority may differ among individual countries (for examples, see Appendix 1) as national legislation or policy may invest this responsibility with a lead ministry or a board/commission representing several ministries. Several levels of oversight and review will most likely be required before bringing the decision to the national level (Section 5. Regulatory frameworks). Thus, the institution conducting the research is expected to have its own independent committees overseeing biosafety and the involvement of human subjects. Intermediate jurisdictional units of government may impose additional levels of regulation.

Results of Phase 3 testing will form the basis for determination as to whether the technology should move into wider scale application as part of a national or regional programme for vector and disease control. The ultimate decision on deployment of GMMs as a public health tool (**Phase 4**) will involve the national regulatory authority, and may additionally involve authorities responsible for determining national or regional disease control priorities (if different from the regulatory body). Phase 4 constitutes an ongoing surveillance phase that will assess effectiveness under operational conditions (both entomological and epidemiological impact), accompanied by monitoring of safety over time and under diverse situations. Long-term surveillance of safety for human health will be

analogous to the pharmacovigilance[8] applied in medicine but, in the case of GMMs, aspects of environmental safety should also be considered. Ongoing monitoring will be aimed at ensuring sustained quality and performance for disease control, and determining whether any changes are needed in management of either the GMM technology itself or other aspects of an integrated control programme. In this regard, it will be important to ensure that a perceived decrease in the disease threat following implementation of GMMs does not lead people living in the area to become complacent and revert to behaviours that could increase transmission pressure.

1.5 Decision-making

In determining whether any GMM technology should move forward from one phase to the next, it is expected that the responsible regulatory authority will take into consideration criteria of both safety and efficacy for its intended use. As described in subsequent sections of this *Guidance Framework*, the transition from one phase to the next will be subject to defined "go/no-go" decision criteria, including efficacy and safety endpoints, and be contingent upon regulatory and ethical approvals.

The meaning of "safe" is not easily defined, as it is recognized that virtually all public health products (including those currently in widespread use against diseases such as malaria and dengue) have some ability to cause adverse effects under certain conditions. Thus, a new product such as GMMs is often assessed in the regulatory review process by determining whether its benefits outweigh its risks.[9] The primary potential benefit of GMMs would be the improvement of human health, and therefore efficacy data will enter into decision-making regarding benefit. The stringency of efficacy demonstration required to judge a new technology worthy of moving forward may well be influenced by the potential for adverse effects associated with the technology, which in turn will differ according to the phase of testing. Variations in individual judgement, as well as the context in which decisions are being made, can lead to differing opinions about risk-benefit assessment. Some might advocate for withholding regulatory approval until absolute assurance of the absence of risk is available, regardless of benefit. However, regulators may feel that other contextual factors also should be taken into account, such as the severity of the health problem addressed by the new technology, and the availability and utility of alternative disease control methods (FDA, 2013). With regard to genetically modified organisms (GMOs), the Nuffield Council on Bioethics has recommended "comparison of the risks of the status quo with those posed by possible paths of action," recognizing that "there can be dangers in inaction, or alternative courses of action, as well as in the adoption of a particular innovation" (Nuffield Council on Bioethics, 2014?).

Other considerations beyond risk-benefit may come into play, especially when decisions are being made to deploy a new technology as part of the national disease control programme (Phase 4). Economic evaluations may be used to compare alternative courses of action as a basis for weighing the options and making sound decisions about investment of scarce resources. Cost-benefit analysis provides for the systematic calculation of benefits and costs in monetary terms and over time.

[8] WHO Pharmacovigilance: http://www.who.int/medicines/areas/quality_safety/safety_efficacy/pharmvigi/en/index.html, accessed 25 May 2014.

[9] For example, FDA (2013), EMA (2011) and Explanation of statutory framework for risk-benefit balancing for public health pesticides: http://epa.gov/pesticides/health/risk-benefit.htm, accessed 25 May 2014.

However, for public health interventions, it may be difficult to calculate the benefits of improved health in financial terms. A related method for comparing the relative costs and outcomes of multiple courses of action is cost-effectiveness analysis, which expresses benefit as a measurement of a particular health gain. For example, cost-effectiveness analysis might allow comparison of alternative malaria or dengue control methods in terms of costs required to achieve a particular reduction in mortality or clinical disease. Public health decision-makers may take a sectoral approach, comparing cost and effectiveness of all possible disease interventions to select a mix that provides maximum health benefits within given resource constraints. Issues that will need to be factored into decision-making include whether the GMM technology will replace or reduce the need for other control measures and, if not, how much the addition of GMMs to ongoing disease control efforts will enhance the overall effectiveness of the programme.

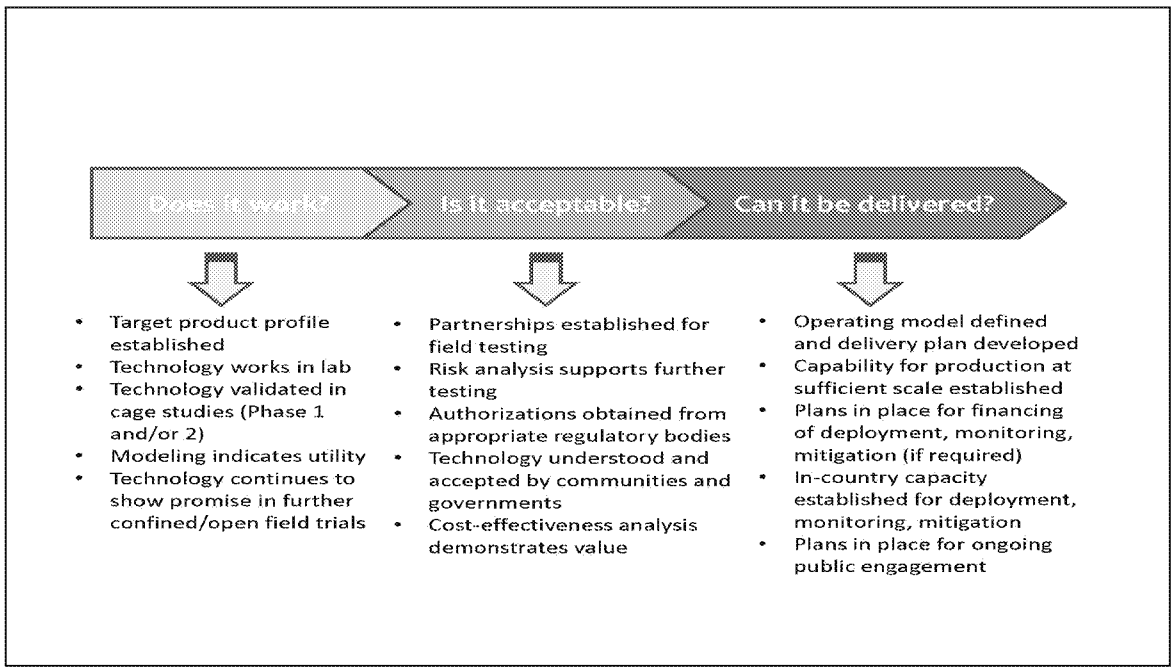
1.6 Critical path for GMM development

Proof of concept for efficacy of the GMM technology is one component of the critical path. Other key elements must be engaged for proof of acceptability as well as proof of deliverability and sustainability (Figure 1.2). Proof of acceptability involves risk analysis, regulatory approval and community/stakeholder authorization. As mentioned, cost-effectiveness of the technology vs. other available disease control methods may influence acceptability. Proof of deliverability involves the development of an operating model with planning for sufficient technical capacity to support wider-scale deployment, production capability at an appropriate scale, financing to support deployment and subsequent monitoring, methods for field-applicable high-throughput monitoring for quality control, management and mitigation capability in case of adverse events, and ongoing stakeholder engagement. Sustainability will have different implications depending on whether the GMM technology is self-limiting or self-sustaining, but in either case an important aspect will include planning the response should indications of resistance to first-generation GMMs be detected during Phase 4 monitoring. As is the case for drugs and insecticides, this may require support for ongoing research to develop next-generation products.

Challenges remain in the identification of a viable model for the development of GMMs as public health tools. Public agencies and philanthropic funders may provide the resources for phases 1 and 2 research. However, the level of support that will be required beyond early, small-scale, Phase 3 testing may be beyond the capacity of such research funders. In the standard business model used for drugs, vaccines and insecticides (including those against malaria and dengue), industry would be expected to pick up a promising lead and provide additional financing for its development into a marketable product. However, GMMs are a new technology primarily being developed for use in low- to middle-income countries and their potential for direct financial returns is uncertain (especially with self-sustaining versions). Small biotechnology companies with limited resources currently represent the only direct industry involved in GMM development. Public-private partnerships, non-profit corporations, and other models of broadly supported funding consortia may provide good precedents for GMM development. Furthermore, technology transfer to disease-endemic countries is an important goal of GMM research.

This G*uidance Framework* focuses primarily on the most immediate issues to be addressed in the critical path to GMM development: proof of efficacy (testing for entomological and epidemiological impact) and acceptability (biosafety, ethics and engagement, and regulatory requirements).

Figure 1.2 Elements of the critical path for GMM development and deployment



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2. Efficacy evaluation

Summary: Both entomological and epidemiological endpoints may be used to test the efficacy of GMMs in reducing morbidity and mortality from vector-borne diseases. The entomological endpoint is a reduction in the likelihood of disease transmission due to mosquito population characteristics, and will be the predominant outcome measure in phases 1–2 and, possibly, early Phase 3, trials. Because this is difficult to measure directly, surrogate indicators may be chosen, and these may include vector population size, transgene frequency, and ability to support pathogen replication and/or GMM fitness. The epidemiological endpoint is a measurable reduction in the incidence of infection or disease in human populations. Epidemiological outcomes will be detected most easily when trials are conducted in high-transmission settings. The specifics of conducting such trials will differ for the malaria and dengue interventions that are the focus of this document. These differences include the fact that persistent endemic transmission locations are available for malaria intervention trials, and therefore effects may be observed more rapidly and unequivocally than in dengue trials, which are likely to be conducted in locations where transmission is more heterogeneous and thus less predictable. Cluster randomized trials offer a powerful design for Phase 3 evaluation of efficacy against disease transmission in field trials. Trial designs must take into account the likelihood of significant seasonal and inter-annual variations. Non-linear relationships between entomological and epidemiological outcomes may also be anticipated. Much of the entomological monitoring required will employ methods used in any vector-control programme. However certain monitoring measures, such as phenotypic stability, will be unique to GMMs. "Go" and "no-go" criteria for moving to the next phase of testing should be determined prior to trials. Specific entomological and epidemiological measures are recommended for each phase of testing.

It is envisaged that GMM strategies will be implemented in area-wide control programmes. These are conducted over large areas that may include several communities and contain at a minimum the generational dispersal range of the target species. Area-wide control depends on the treatment of such large regions for success, particularly in situations where effectiveness of the control measure will be influenced by the potential for reinvasion. This implementation scale stands in contrast to interventions such as repellents or nets that are effective at both household and individual levels. Thus, the scale of testing and exposure of entire populations to GMM interventions have implications for how trials can be conducted. Preliminary experiments can be conducted in laboratories and outdoor cages, but testing during phases 1–3 proceeds through increasingly larger scale (Figure 1.1), ultimately to open-field releases in which the efficacy of the technology can be assessed most realistically. The purpose of any open-release experiments should be clear and experimental protocols should be made available in advance.

While GMM technology has not yet been tested extensively in the field, experience gained from conventional mosquito control programmes using methods such as indoor residual insecticide spraying, outdoor space spraying and larviciding can help predict its efficacy. Experience from sterile insect control programmes on agricultural pests will also be helpful in predicting outcomes, since population suppression or preventive releases are the most immediate aims of planned genetic mosquito control. Although conventional insecticidal control is usually not species-specific, its

effects are similar to self-limiting GMMs in that they are not permanent. This self-limiting nature provides a degree of intrinsic safety, in that implementation can be halted to mitigate and, possibly, reverse adverse effects.

This chapter focuses on three key issues of efficacy evaluation: 1) the definition of entomological and epidemiological efficacy endpoints of GMMs; 2) methodology issues and considerations related to empirical measurement of efficacy; and 3) empirical measures of efficacy in the four different development phases. This guidance relates to malaria and dengue vectors, as development of these applications is currently the most advanced and their biology represents many other vector-borne disease systems. Other disease vectors also may become targets of GMM control, but details for determining their efficacy will not be discussed specifically.

Feasible applications of GMMs that will not be addressed in this section include those in which mosquito control agencies might want to use GMMs against the threat of disease or introduction of a vector. For example, such a preventative release is used in California and Florida, USA, where exclusion is accomplished by conventional SIT programmes against Mediterranean fruit flies.[10] Powerful population suppression by GMM strategies could find a market against pest mosquitoes in mosquito control programmes, even where disease transmission is not a major consideration. In such cases, the entomological outcome of the frequency and scale of target species outbreaks would be sufficient to demonstrate efficacy. Similarly, the release of GMMs containing drive mechanisms to spread refractoriness in a population might be used to preclude the onset of transmission. If such protection were inexpensive, stable and acceptable, it might be implemented with minimal proof of efficacy against disease.

2.1 Efficacy end points of GMMs

The efficacy measurements of GMMs can be defined by entomological and epidemiological outcomes. These differ according to the disease, the vector species and the epidemiological circumstances. Endemic disease situations are common for malaria and the effects of interventions during trials conducted in such locations may be determined more rapidly than for dengue, which is often spatially and temporally heterogeneous. These differences, as well as the occurrence of multiple vectors in one place (particularly for malaria) determine the measures of efficacy that are appropriate and feasible. Researchers planning trials must consider not only what is ideal, but also whether field sites are available for determining specific epidemiological outcomes using the most powerful protocols.

The epidemiological endpoint is a reduction in infection or clinical disease incidence

In trials designed to prove epidemiological impact, reductions may be measured by various means including infection incidence, clinical disease incidence or prevalence of infection in at-risk populations. In general, trials designed to detect a decrease in the incidence of infection will be able to achieve a statistically meaningful result with a smaller cohort size than trials that measure decreased incidence of disease, since only a subset of those infected may develop overt disease.

[10] USDA-CDFA Mediterranean Fruit Fly Exclusion Program: http://www.cdfa.ca.gov/phpps/pdep/prpinfo/, accessed 25 May 2014.

Reduced infection incidence is generally expected to result in decreased mortality and morbidity, although this will not always be the case; for example, during resurgence of disease in a naïve human population, unusually high rates of morbidity and mortality may occur. Multi-year data collection may be required to demonstrate positive effects where disease is epidemic, highly variable from year to year or of low prevalence. Pre-existing immunity to pathogens and viruses also may influence measures of efficacy and must be considered in the experimental design.

The entomological endpoint is a reduction in the likelihood of disease transmission due to mosquito population characteristics

The entomological measure of transmission (also called "force" or "intensity") due to mosquito population characteristics is the entomological inoculation rate (EIR). EIR describes the degree of infection risk that a human population is exposed to for a particular disease as determined by assessing the vector mosquito population. EIR would be a distribution of frequencies of infectious bites over time for a range of people with different demographic characteristics in the area. A control programme would shift this distribution to a lower mean frequency, but the shift might be more or less for different demographic groups. EIR is influenced by several factors that are specific to the geographic area, including climate, bionomics of local vectors and socioeconomic factors. Accurate measures of EIR are most easily made when the prevalence of a pathogen is high – hyperendemic disease transmission scenarios – and most difficult when prevalence is low or in epidemic situations. It should also be anticipated that the level of disease transmission might change during trials for reasons unrelated to the trial itself, unusual weather that affects vector abundance being the most common influence. Researchers designing the trial should prepare for such eventualities by proposing variations of the protocols during the planning phase and considering the need for adaptive management during the trial (assuming this is acceptable to regulatory authorities). The EIR varies widely in time and space in regions of epidemic transmission, and its direct determination will seldom be feasible. In practice, its measurement requires analysis of field-collected mosquitoes – often in large numbers and over long periods of time – for the presence of infective pathogens, so it can be determined only in the presence of at-risk human populations.

While a measured reduction in the EIR is the most desirable of entomological outcomes, demonstrating this will be difficult or impossible during confined Phase 2 and many Phase 3 trials. This difficulty will be particularly great when there is the potential for substantial heterogeneity in transmission, as is common for dengue. Furthermore, it is anticipated that ideal testing locations for GMMs will be chosen in part for their confinement characteristics (ecological or physical islands), and the number of vector species present. These specifications will limit further the range of transmission scenarios and specific field sites that are available.

For these reasons, it is necessary during phases 1 and 2 to infer reductions in EIR by surrogate vector indicators that contribute to the EIR. These may include daily survival, changes in absolute density, altered propensity for feeding on humans, frequency of anti-pathogen effector genes and intrinsic competence for developing infection. These indicators can be measured directly or calculated from measurable data, e.g. the realized frequency of an anti-pathogen effector phenotype in a population or the rate of spread of a transgene. The specific characteristics of GMMs must also be considered in determining which indicators will be most useful to measure. For example, the frequency of GMMs

that suppress populations in part by providing larval competition before the lethal effect occurs may have different effects on adult abundance from GMMs that produce no progeny. Therefore, monitoring larval transgene frequency and egg number have predictive value but hatching rate is less diagnostic.

Beginning in Phase 2, feeding of mosquitoes using blood from infected persons in contained conditions may provide a useful indicator if the GMMs are expected to have reduced intrinsic competence to support pathogen replication. Such tractable measures then can be used to parameterize models to predict the potential effect on EIR under various transmission conditions. Carefully measuring these during phases 1 and 2, and integrating the outcomes into transmission models, is an essential part of predicting efficacy. Use of surrogate efficacy measures may be necessary even during Phase 3, and will help to determine the need to move to large trials for epidemiological endpoints.

2.2 Empirical measures of GMM efficacy

Trials must be designed to allow measurable reductions in the incidence of infection

The measurable epidemiological outcomes, reduction in the incidence of infection or disease in human populations, are few relative to the various GMM technologies that may be undertaken to accomplish them. Therefore, considerations for measuring these outcomes are discussed before proceeding to the variety of entomological measures and considerations of efficacy that will apply to population suppression and replacement strategies. Differences in detection and transmission dynamics between malaria and dengue will be discussed separately after commonalities are described. The endpoints for either disease in the context of GMM applications are similar, but the means by which these can be measured differ.

A statistically sound epidemiological trial design must be selected

The cluster randomized trial, (Hayes et al., 2000), in which groups of people are evaluated (as opposed to individuals), is anticipated to be the most powerful design for detecting the efficacy of GMM applications in Phase 3 trials when an epidemiological outcome will be measured (Wolbers et al., 2012). Longitudinal studies with enrolled cohorts are recommended to determine infection incidence. Passive case detection may be implemented for each cluster to determine the effect on clinical disease incidence; however active case detection is preferred whenever resources are available. The most accepted malaria[11] and dengue fever[12] case definitions should be used. Good clinical practice (GCP) should be followed (EMA, 2002).

Careful site selection increases the likelihood of detecting significant results

[11] Centers for Disease Control and Prevention. National Notifiable Diseases Surveillance System (NNDSS). Malaria 2010 case definition: http://wwwn.cdc.gov/nndss/script/casedef.aspx?CondYrID=759&DatePub=1/1/2010%2012:00:00%20AM, accessed 25 May 2014.

[12] Centers for Disease Control and Prevention. Dengue. Clinical description for case definitions: http://www.cdc.gov/dengue/clinicalLab/caseDef.html, accessed 25 May 2014.

Detecting statistically significant reductions in epidemiological measurements would require a large number of clusters that may not be feasible in sites with low infection or incidence of clinical disease. Therefore, particularly for malaria, which often occurs at high EIR, trials in endemic areas are recommended. It is considered likely that a GMM intervention that is effective in an endemic area will also be effective in lower transmission conditions although the reverse cannot be assured. Phase 2 and 3 trials should aim to detect an effect in one transmission season. Because dengue and malaria transmission vary from year to year, multi-year trials may be necessary to ensure that both low- and high-transmission years are included in the study.

Mosquitoes disperse locally, but long distance movement by malaria and dengue vectors unaided by human activities or large weather events has not been observed (Service, 1997). However, movement of mosquitoes can confound the interpretation of releases and prevent a positive trial outcome both by immigration of wild mosquitoes and emigration of GMMs. When wild mosquitoes move from untreated areas into treatment areas, the degree of sexual sterility or increase in transgene frequency will be reduced relative to that that would be achieved in closed populations. In contrast, a self-sustaining drive mechanism with intergenerational effects may spread a gene well beyond the site of introduction and contamination of control areas must be prevented or accommodated in the trial design. Therefore, effects will be demonstrated most easily when repopulation of treatment areas by untreated wild mosquitoes and dilution of the GMM is minimized by strong isolating factors. If the GMM is a rapidly self-limiting one, separation by two kilometres will probably be sufficient (Service, 1997), but if a self-sustaining GMM is being tested, separation distances must be greater in proportion to the expected rate of drive. Thus, the clusters for both types of technologies must be sufficiently isolated so that the GMMs are confined to, and excluded from, experimental and control clusters, respectively. Physical or ecological islands, or sufficient geographical distances, may prevent results from being confounded by inadvertent cluster contamination. Measurements of dispersal (commonly determined directly by mark-release-recapture or estimated from population genetic studies) and previous studies can guide the selection of conditions that provide sufficient isolation for various GMMs, and these must be confirmed prior to trials. GMMs that contain genes encoding visible markers such as fluorescent proteins can be distinguished easily from wild-type mosquitoes. Large-scale gene amplification technologies to detect a molecular marker are also feasible. Other temporary markers such as fluorescent powders can also be useful to distinguish dispersal when populations already include GMMs.

Ongoing disease control measures must be considered

Phase 2 confined-field trials and Phase 3 open-field trials will probably use GMMs as a part of an integrated vector management (IVM) programme. Therefore, the effect of ongoing control measures on the outcomes of the GMM trials must be considered. It is neither experimentally necessary nor ethically acceptable to test GMMs under conditions in which ongoing vector control activities are not continued. Therefore, site evaluation should include entomologically and epidemiologically similar field sites in which the same standard of care is being applied. Likewise, it also is necessary to continue any control activities being conducted when CRTs begin and to ensure that they are applied uniformly across sites. A change in the use of conventional control methods during testing could change the transmission dynamics on which trial design was based. For

example, this might be the case if those living in the trial site stop practicing other avoidance measures because they perceive a diminished threat. Thus, there are both scientific and ethical reasons to ensure that the trial is understood to be a research effort with no guarantee of protective effect. Alternatively, such a change could occur if a new control measure is introduced into routine use at the trial site, so it is important to coordinate as closely as possible with the regional vector control programme during trial planning and implementation.

GMMs are expected to be compatible with conventional control measures unless those measures exploit some weakness peculiar to the GMMs (Alphey et al., 2010). For example, if high levels of insecticide resistance occur in wild populations and the GMMs are susceptible, then continued use of the specific insecticide(s) to which the wild population is resistant will disproportionately affect GMMs and diminish or nullify their effects. Therefore, considerable thought should be given to the phenotypes of wild mosquitoes and GMMs, the control measures that will be applied for CRT site selection, and potential vector control mitigation before making final choices.

Attention should also be given to ensuring that no major differences exist in individual human behaviour between clusters or trial sites that may affect the intervention (WHO, 1997) e.g. the use of personal protection measures (including mosquito nets), the domestic use of insecticides, occupational exposures, and migration and human movement between treated and untreated communities. Information may be obtained through interviews that may be supplemented by direct observation (e.g. of anti-malarials, bed nets or insecticides available in the home). For lengthy trials, consideration must be given to the potential that new control measures (e.g. vaccines) may become available, and decisions made in collaboration with public health officials about how such a situation might be handled.

Comparative efficacy between GMM and conventional vector control

Ultimately, GMMs may be considered as a substitute for conventional vector control (e.g. insect-treated bed nets – ITNs – indoor residual spraying – IRS – or environmental management) if there is evidence that such modification may be more cost-effective or more environmentally favourable relative to existing control measures. Alternatively, GMMs may be combined with conventional vector control if the methods are complementary and synergistic effects are anticipated. The synergistic effect of combinations of two vector control methods can be determined if one treatment area is subject to both methods and the control area utilizes only conventional vector control. To compare the efficacy of GMMs and conventional vector control, a Phase 3 trial design should include GMMs as one arm and conventional vector control as the other arm. However, design of such comparison trials must be considered carefully to ensure that the population in the GMM arm is not subjected to unnecessary risk in the absence of standard control methods. Such trials should be justified by adequate prior demonstration of GMM efficacy. Phase 3 entomological and epidemiological endpoints described above should be measured. An appropriate number of clusters should be used to allow sufficient statistical power to detect differences. Cost-effectiveness analysis of GMM or conventional vector control, or a combination of the two methods, should be performed.

Special considerations for trials of dengue interventions

Since dengue transmission is highly variable, it is likely that trials must be conducted on large spatial and temporal scales, with large numbers of clusters, in order to detect an epidemiological effect. Large reductions of normally high transmission could easily be measured. But, more typically, even a GMM trial that completely eliminates transmission might need to extend over several years to provide sufficient statistical power to conclude efficacy. GMM technologies are designed to reduce the likelihood of transmission for people within the area under management, rather than treat individuals within it. Thus, the area should be large enough so that large numbers of individuals are not being exposed routinely to unknown risk of infection when travelling outside their respective control or treated area, which could confound interpretation of trial results. Ideally, trial planning will include methods to allow individuals becoming infected outside of the trial area to be identified so that their contribution to incidence can be discounted. The trial plan also should anticipate variation in transmission levels that may necessitate changing the scope of the trial (for example, Phillips-Howard et al., 2003).

A reduction in the incidence of clinical disease may be a possible measure of efficacy when dengue transmission is high. An alternative method, which is likely to be more feasible, given the expected heterogeneity of transmission, will be to measure the frequency of individuals positive for dengue antibodies in blood samples (Endy et al., 2008). In areas where the incidence is low, reduction in dengue virus-specific IgM[13] and/or IgG[14] antibodies obtained by sero-survey can provide an effective epidemiological endpoint. Performance of serological plaque reduction and neutralization assays in a longitudinal cohort trial, accompanied with active surveillance for virus recovery on a subgroup of people with clinically-apparent infection, may allow more accurate information on dengue risk. The need to evaluate impact on the four different dengue virus serotypes must be kept in mind.

Where regional dengue transmission is due to a single vector species, if GMMs are effective, and achieve and maintain local elimination of that vector, then it may be unnecessary to demonstrate epidemiological outcomes as a determinant of GMM efficacy. In such a case, vector elimination can be used as the efficacy measurement. However, vector abundance *reduction* does not necessarily translate directly into reduction of dengue incidence, as transmission has been observed in the presence of low apparent numbers of mosquitoes. Determination of the threshold of vector abundance reduction required to achieve significant reduction in dengue disease incidence requires epidemiological modelling and empirical studies, and such threshold vector densities may vary between geographical localities. In the case of vector population replacement by GMMs, measurement of infection or disease incidence reduction relative to untreated controls, despite being costly, may be necessary to provide high confidence in the efficacy of this novel GMM strategy.

Special considerations for trials of malaria interventions

The high levels of malaria transmission encountered in much of sub-Saharan Africa mean that measuring epidemiological outcomes may be relatively easier for malaria than for dengue. However, designation of epidemiological endpoints for malaria must take into account the multiplicity of

[13] Immunoglobulin M.

[14] Immunoglobulin A.

vector species and, to a lesser extent, parasites. Identifying appropriate trial sites may be challenging. Efforts should be made to find sites matched for human demographics and disease patterns, and to ensure sufficient confinement to satisfy the requirements of RA and trial design. The number of vector species responsible for transmission and their ecological interactions must also be considered.

Several methods are available for malaria diagnosis.[15] Historically, the "gold standard" has been microscopic examination of blood smears. However, many rural clinics lack necessary microscopes and trained personnel for malaria diagnosis. Consequently, the non-microscopic rapid diagnostic tests (RDTs) have become popular in various endemic settings. Many malaria RDTs are available commercially from several manufacturers.[16] The specificity of the tests is variable; some can only detect *P. falciparum*, while others also can detect non-*P. falciparum* infections. For applications under field conditions, RDTs must be stable, simple to use, easy to interpret, and sensitive to clinical malaria cases. The commonly recommended lower detection limit for *P. falciparum* infection is ~100 parasites/µl of blood. The specific RDT for malaria diagnosis used in a trial must be selected carefully and evaluated thoroughly according to WHO guidelines.

Most malarious areas contain one or two dominant vector species, and it may be difficult or impossible to restrict testing of GMMs to sites containing only the target mosquito. If single-vector sites are used for trials, the results may not be generally applicable. However, it is clearly not feasible to determine epidemiological efficacy accurately during phases 2 and 3 by targeting a single species when it is well established that numerous other vectors of the same pathogen are present and are sufficiently abundant to maintain high levels of transmission.

Experiments and modelling should be conducted prior to GMM field testing to determine in which seasons and ecological contexts the GMMs have a reasonable chance of affecting epidemiological outcomes. For example, preliminary experiments or historical records may reveal the contributions of individual vector species to the overall disease transmission levels. While these are often considered additive, each species' contribution may not conform to such a simple relationship, especially when the efficiency (vectorial capacity) of one key vector species is much higher than others. Furthermore, there is a possibility that suppression of one target species could cause niche replacement by other, closely related vector species. Interpretation of epidemiological outcomes by GMMs in multi-species sites requires caution. These issues should be anticipated as early as possible, and factored in to the choice of target species in GMM design and selection of trial sites when entering into field testing.

Entomological efficacy must be determined in the context of the anticipated use of the GMM technology

Few GMM interventions will be implemented in isolation, thus their performance will be determined best in the presence of other anticipated control measures. Indeed, it is an accepted procedure to conduct efficacy trials for new products in the presence of the standard of care for disease control in the area. If the anticipated use of GMMs is to further reduce or eliminate populations that have

[15] WHO malaria diagnostic testing: http://www.who.int/malaria/areas/diagnosis/en/, accessed 25 May 2014.
[16] WHO/TDR malaria rapid diagnosis work: http://www.wpro.who.int/sites/rdt, accessed 25 May 2014.

been suppressed by seasonal depression or conventional methods, then the efficacy of the GMMs should be evaluated in that context. If the intended use of GMMs is to replace the conventional control methods, the cost-effectiveness and reliability of the GMMs needs to be compared with these methods. The reliability of the GMMs as a component of the suite of interventions is a central consideration. Particularly for developing countries, GMMs that are highly effective under ideal circumstances will be less attractive if they perform poorly when logistical, management or ecological difficulties arise and are common. The ability to provide for the ongoing cost of an intervention should be a consideration.

The specific experimental designs to be used may vary widely according to the specific mosquito, study site and country, and the progression of experiments from the laboratory to the field will require reconsideration at each stage. When possible, the validity of a specific experimental design should be assessed during the process of peer review. In non-academic circumstances where funding does not ordinarily require peer review, independent review by experts is highly recommended.

Surrogate endpoints must be chosen for early phase testing

GMM strains are built for specific circumstances where their potential for reducing EIR has been investigated and predicted with mathematical models. These models highlight key performance characteristics that then can be measured in the laboratory to the necessary precision as a first approximation of field performance. The performance characteristics vary with the specific strategy but include population suppression, appearance of sexual sterility, mating competitiveness, spread rate and frequency of a transgene in a population, and appearance of a particular phenotype. Measurement of entomological surrogate indicators for EIR requires close supervision and dedicated well-trained staff. In the case of self-limiting population suppression, vector abundance and its effect on EIR are the most direct measures of entomological efficacy and there are standard methods available to determine them (WHO, 1975; Silver, 2008).

During the course of the trials, experimental outcomes should be used to redefine the parameters of the intervention's computer models. These changes may require alterations to the trial design or the outcomes that can be expected. Model performance should also be monitored during the trials to determine whether its predictions are validated by trial observations. Stakeholders and regulators should also be clearly informed on how modified model predictions may affect trial conduct or continuation.

The influence of seasonal and inter-annual variations on trial design must be considered

Seasonal and inter-annual variations in climatic conditions and other intervention measures that affect vector abundance, species composition, transmission intensity and disease incidence are common. Phase 2 GMM trials that involve small-scale ecologically confined field releases, and Phase 3 testing that involves large-scale open-field releases, should take these variations into consideration to ensure experimental success and to enable the results to be generalized.

Self-limiting population reduction GMMs will require regularly scheduled releases, and within a short-term trial a reduction of the population size could be a fortuitous characteristic of a specific

season alone, but one that might not be repeatable. Multi-year evaluations would provide more robust assessments of both the climate and co-intervention effects, as well as an idea of how the intervention effect varies as a function of annual medium-term variations.

Population replacement in which a gene drive system is involved may take several years after repeated releases to increase the frequency of refractory alleles to an effective level. In this case, mathematical modelling should be conducted to predict the necessary trial duration for evaluating efficacy. Uncertainties, assumptions and unknowns in disease transmission models and vector bionomics should be transparent, and a variety of models and scenarios should be considered, model parameter uncertainty explored, assumptions tested and model predictions validated at each stage.

Non-linear relationships between entomological and epidemiological outcomes can be expected

The simplest outcomes to measure when GMM sterile-male methods are used are reductions in female fertility. This is typically determined by a direct measure of the number of larvae produced per female, and can be performed using laboratory-reared mosquitoes or by obtaining eggs from blood-fed field-collected females. While it may seem that increases in sterility would lead to reductions in adult populations, there is seldom a direct relationship due to the dynamic nature of larval competition. Two kinds of effects are expected: (1) negative density dependence[17] (Juliano, 2007; 2009) is common and will tend to dampen the initial effects of reduced fecundity on adult population sizes. These interactions mean that different GMM self-limiting male sterility approaches will perform differently (Yakob & Bonsall, 2009). (2) Over-compensation[18] under some circumstances may cause increases in the adult population size when larval density decreases. Both of these effects occur due to competition for food in larval sites. Knowledge of the population dynamics as determined by larval abundance would be a useful predictor of the levels of releases and sexual sterility that will be necessary in order to realize particular levels of population suppression. Ecological studies prior to releases should be performed to determine the characteristics of sites and predict the usefulness of GMM interventions.

Reductions in vector abundance or increases in refractory transgenes to a high frequency should lead to a reduced EIR. In the particular case of malaria in hyperendemic areas, this desirable entomological outcome is expected to result in reduction of disease only when EIR falls below a threshold necessary to maintain transmission, often cited as one infective bite per year (Shaukat, Breman & McKenzie, 2010). In such areas, a substantial reduction in transmission intensity by the GMMs or combination of interventions will probably be needed to demonstrate an epidemiological impact.

Entomological monitoring unique to GMMs

Most of the characteristics used to monitor GMM functionality are not unique to the technology. Methods to evaluate these characteristics have been developed and are used routinely to gather

[17] Population regulation in which increased population density reduces its rate of increase. In this case, adding more immature individuals to a population does not proportionally increase the number of adults.
[18] Population regulation in which reductions in some stage of the population actually increase population size, e.g. by improving survival to adulthood.

entomological data. These include determining adult abundance, host preference and/or the ability to develop and transmit parasites or viruses. These and other biological characteristics should be catalogued thoroughly during GMM testing. GMM production should utilize standard operating procedures (SOPs) and good manufacturing practices (WHO, 1992). Reproducible life history and phenotype can only be expected if the mosquitoes are reared and maintained using standardized procedures.

Molecular properties

A thorough description of the GMM describes the transgene components, genetic background and novel phenotypes. This description allows preliminary assessment of the GMM itself and observations of changes in salient features, including the transgene sequence, its insertion site and strain background. The description of the GMM should include information about the strains that contributed genetic material.

Phenotypic stability

Among the few characteristics of GMMs that are unlike those monitored for typical entomological surveys, phenotypic stability is paramount and is a strong determinant of efficacy. This can be evaluated by answering several questions: does the mosquito exhibit the design characteristics in both laboratory studies and field simulations? If the phenotype is not fully penetrant[19] but the transgene is stable, what effect on its efficacy and fitness do models predict? It will be possible to measure stability in increasingly realistic GMM trials as they move forward through the phases; however, the process should begin in Phase 1. The genetic diversity of the mosquitoes and pathogens with which the GMMs interact, and the environmental variations, will increase and may reveal novel variations in phenotype expression as advanced phases of testing become more realistic in Phase 2 and Phase 3 trials. Such measurements should continue periodically in the context of a post-implementation surveillance (Phase 4).

Variations in expression of a transgene should be quantified so that significant deviations in novel environments can be detected. It is particularly important to determine whether the phenotypes that have been measured in stable laboratory environments are consistent when, for example, temperature variations are experienced. Similarly, laboratory evaluations should include transgene expression in aged individuals and in a variety of genetic backgrounds. If expression of the phenotype is conditional on some environmental factor, the effects of variation in the presence of that factor should be examined.

Loss of phenotypic expression can result even in the absence of transgene mutation and can negatively affect efficacy. Evolution of resistance to a transgene effector can occur either in the GMM strain itself (phenotypic drift or gene interaction) or in the target mosquito population following lengthy exposure. As with resistance to insecticides, this is extremely difficult to predict with high certainty from small laboratory studies, but one can measure pre-existing resistance in the target population and then monitor the phenotype in the field over time. As is evident with

[19] The transgene phenotype is predictably absent in some proportion of the individuals in a population despite the transgene being present in an unmodified form in all individuals.

insecticide resistance, it is not the appearance of resistance but its frequency that mitigates the usefulness of the intervention. The likelihood of such resistance and its consequences should be considered thoroughly and measures put in place as part of the trial plan to prevent (if possible), detect and respond to it. Preliminary laboratory examination of the likelihood of resistance arising may in some cases be possible and this consideration should be part of the early RA (Section 3. Biosafety). As described above for instability related to mutation, these effects can be expected to become more evident during phases 2 and 3. Measuring such effects should be intensified beginning with confined Phase 2 trials while unanticipated effects can be restricted in time and space. The pathogen also has the potential to develop mechanisms for evading refractoriness of GMMs in the case of population replacement. Thus, during phases 3 and 4, refractoriness of GMMs to pathogen should be carefully monitored.

Fitness

"Fitness" of transgenic mosquitoes has been the subject of much study and discussion (Catteruccia, Godfray & Crisanti, 2003; Irvin et al., 2004; Moreira et al., 2004; Marrelli et al., 2006; Li et al., 2008; Amenya et al., 2010; Isaacs et al., 2012). While this is a characteristic relevant to long-term population trends, it is of less relevance to self-limiting population suppression strategies: the mosquitoes used for the latter approaches have reduced fitness by design. What is relevant is their ability to suppress wild populations and, for GMMs intended to have a multigenerational effect (sex-ratio distortion[20] or inherited sex-specific sterility), the duration of the suppressive function. One measure of the maximal rate of effect on population suppression is the mating competitiveness value (Fried, 1971). It indicates (usually on a 0–1 scale) the relative frequency of mating of a male in question (in this case, GMMs) when in competition with a reference wild-type male. However, there is no absolute value of competitiveness that precludes the use of a strain since even very low-value insects (e.g. 0.2 for Med fly) can effectively suppress populations if sufficient numbers are released. Nonetheless, measuring competitiveness, longevity and the duration of effect will provide indices that determine the necessary scale of releases and their efficiency and are, therefore, important for strain efficacy evaluation.

In contrast, the fitness of the GMMs used in population replacement and self-sustaining approaches is critical, specifically, the effect on fitness due to the transgene expressing the desired phenotype. The designed effect is not population replacement per se, but rather the introgression of a transgene causing a phenotypic change into an otherwise wild mosquito population. After release, recombination between the transgene and the wild genome will occur at rates determined in large part by the presence of natural inversions and homologous pairing. Therefore, the fitness of repeatedly out-crossed mosquitoes must be measured. Assuming that a transgene is in a drive system, the loss of fitness and reduction in gene frequency due to the transgene must be compared to hyper-Mendelian inheritance rates[21] due to the drive mechanism. Models can be used to predict the ranges of fitness and drive that will permit transgene spread. When a gene drive system is

[20] Changing the sex ratio among progeny from the typical equal numbers of males and females to progeny consisting largely of males.

[21] An individual heterozygous for a transgene will produce progeny that are 50% transgenic in a normal non-drive system. Hyper-Mendelian inheritance is expected in drive systems, and these individuals produce > 50% transgenic progeny.

implemented to achieve population replacement and self-sustaining strategies, the frequency of the functional gene in mosquito populations into which the GMM has been released is the ultimate measure of this balance. While such measures can be used to refine efficacy predictions in Phase 1 testing, Phase 2 and 3 trials are necessary to develop final measures. This is because the activity of the transgene can be expected to differ depending on the genetic backgrounds in which it occurs.

A reduction in the EIR is the ultimate result of successful self-sustaining approaches. Even these kinds of GMMs are likely to require multiple releases over a large area and for long enough to establish the transgene at a frequency in the population high enough to achieve the desired effect. When a GMM is implemented by such multiple releases, it is of little value to conclude effectiveness based on more limited trials. For some interventions, this will necessarily increase the scale of testing required before the potential of the technology can be assessed – a requirement that should be taken into account in RA.

Independent verification of results will increase confidence

All novel vector interventions are open to critical scrutiny until their value has been demonstrated. Similarly, trials of GMMs may be controversial, and even positive results may be questioned if the research team involved is the only one to document the methods and results. Research teams should strongly consider establishing an independent monitoring body to validate and interpret the results , as is routinely the case for clinical trials:

> An independent Data and Safety Monitoring Board (DSMB), including a clinical monitor should be appointed for the trial (see Smith and Morrow, 1996). This should be an independent group that can testify that the trial protocol has been properly followed and that relevant quality control procedures have been operating for the duration of the trial. This Board should be set up before the trial begins rather than once it has started, as unfortunately is often the case (also trials in which this has not been done have often been those which have given rise to greater controversy). (WHO, 1997.)

Methods to ensure transparency and independent validation of results should be considered during the trial design, but careful thought should be given to whether a DSMB is necessary for trials that do not include epidemiological outcomes. Simpler but widely accepted[22] alternatives (i.e. an independent monitor or an oversight panel) may be designed for entomological outcome trials, which could be tasked with particular activities that are a subset of the full trial audit but whose scope is adequate to maintain independence and validation. The expertise of those chosen for this role must adequately represent the knowledge to understand and analyse trial conduct and the appropriate trial outcomes such as vector ecology, behaviour, and population genetics and biology. The selection of individual(s) for this task should be a transparent process. They should not only provide the appropriate expertise, but should also be free of conflict of interest on the trials' outcomes.

[22] For example, the National Institute of Neurological Disorders and Stroke (NINDS) guidelines for data and safety monitoring in clinical trials: http://www.ninds.nih.gov/research/clinical_research/policies/data_safety_monitoring.htm, accessed 25 May 2014.

"Go" and "no-go" criteria must be determined prior to trials

Transition from the laboratory to the field should always be planned with clearly stated performance milestones at which point the project either proceeds to the next level, moves sideways to determine whether the unmet milestone is due to an artifact or experimental design issue, or the trial is discontinued. For cage studies where population suppression or an increase in transgene prevalence is the goal, the researchers must establish clear ranges of performance that warrant proceeding. The oversight panel should independently assess these performance standards. Performance ranges can be informed by modelling the GMM performance characteristics that must be met in order to achieve the desired outcome in the anticipated ecological and geographical context at the next (initially entomological) level of testing.

The consequences of trials become greater as they move from physically confined to ecologically confined and open-field release. Monitoring to detect adverse effects must increase accordingly. Whereas under physical confinement, unproductive effort will likely be the only "hazard" of unnecessarily extended trials, human and environmental hazards must be evaluated as GMM trials move to field release. These challenges are discussed in Section 3: Biosafety.

There are four definite "no-go" points relative to both efficacy and safety criteria: 1) unanticipated disease transmission outcomes linked to the experiments; 2) an unanticipated environmental harm results from the experiments; 3) political or social opposition or unrest prevents the safe continuation of the trials; and 4) the phenotype of the GMM deviates significantly from the one intended. Depending on the technology, the fourth example could include: loss of sexual sterility, high rates of refractoriness failure, or deviations from expected sex ratios. In addition to a no-go trigger, remediation plans should be in place for such events (Section 3. Biosafety).

If no negative effect on human health or environmental quality is determined to result from unsuccessful trials, assessment by the relevant national authority and donor of the value of proceeding will determine whether the project should continue. It is common for sterile insect technique programmes to evolve methodologically during production and release start-up, so initial failure is surprising. The technology developers may make a persuasive case that failures were due, for example, to mosquito production failures, or unusual weather or implementation problems. In such a case, lack of efficacy does not require a no-go decision, but could preclude moving to the next phase until the cause of the failure is clarified and corrected.

2.3 Recommendations for efficacy measurements at different GMM testing phases

The final section of this guidance presents some recommended experimental activities for efficacy evaluation of GMMs in different testing phases. It is likely that GMMs will be used in the absence of other control methods in Phase 1 and large out-door cage testing of Phase 2. Conventional experimental approaches involving direct comparison between GMM cages and control cages with random treatment assignment may be used. In this case, only entomological measurements can be made and, thus, the primary objective should be the potential for reducing transmission intensity as

indicated by entomological surrogates. A sufficient number of replicates should be used to detect the expected difference in the entomological outcomes between GMM and control cages.

Efficacy measurements will vary depending on the intended effects of GMM strategies and testing phases. It is expected that measurements of epidemiological outcomes will not be undertaken until entomological outcomes clearly predict a reduction in the EIR. For example, transmission intensity cannot be measured in Phase 1 testing in a small-scale laboratory setting or in larger population cages. Instead, transgene phenotype stability, population reduction, and transgene spread and frequency are feasible, and are meaningful indicators of GMM efficacy. These must be considered within the context of the disease transmission setting in which the GMMs will be tested and/or deployed.

Initially only entomological outcomes will be possible to measure: many of these must be monitored throughout the phases of development. As testing moves to settings in which humans are, or may be, present, increased attention to epidemiological outcomes must be added. For example, for GMM strategies aimed only at population suppression, including self-sustaining sex-ratio distortion or sterility factors, one can measure vector population reduction or sex ratio during phases 1 and 2 (physical confinement) and it will only be possible to add measures of transmission risk after field releases commence. Alternatively, initial GMM strategies aiming at population replacement will only be able to use measurements such as transgene stability and frequency before adding EIR reduction in later phases.

The following section catalogues typical measurements and designs that should be considered to determine efficacy. Additional recommendations for conducting Phase 1 and Phase 2 physically confined trials of GMMs with a gene drive system have already been published (Benedict et al., 2008). The priority of various activities will change as experience and knowledge about performance characteristics in diverse settings is gained, but thorough strain description is an important activity to begin early in development regardless of the GMM type.

Phase 1. Laboratory population studies

Only entomological outcomes can be determined in Phase 1. Pathogen interactions can, however, be measured.

- Basic description of the transgene, including its sequence, insertion site, phenotype and inheritance. This information will be used during phases 2 and 3 to confirm the GMM's characteristics.
- Stability of the transgene and its phenotype.
- Life-history characteristics in controlled environments.
- Mating competitiveness against laboratory mosquito strains.
- Frequency of GMMs that express the desired characteristic and the level of expression.
- Capability to host and transmit pathogen isolates.
- For drive systems, rate of spread of a transgene in laboratory cage populations.
- For population suppression strategies, rate of suppression in laboratory cage trials.

- Mating frequencies and egg hatching rates within the strain and in crosses to laboratory strains.
- GMM release simulations in large indoor cages.
- Modelling effects anticipated in wild populations.
- Establishment of SOPs for GMM production and release.

Phase 2. Physically and ecologically confined field trials

Physically confined, or "contained," refers to trials performed in large outdoor cages from which escape is highly unlikely due to physical barriers and special procedures. Such trials allow rapid termination and simple detection of escapees. "Ecologically confined" refers to those trials conducted in delimited areas from which escape is unlikely due to some ecological or geographical isolating factor. These include ecological or physical islands. Regulators will determine whether both types of trials are necessary, a decision that may be determined more by safety rather than by efficacy considerations. Epidemiological outcomes may begin to be measured in confined release trials, although, for the reasons explained above, this will be uncommon due to the small scale of the trials.

Entomological activities in physical confinement

- Mating competitiveness against mosquito strains having a wild[23] genetic constitution.
- Frequency of GMMs that express the desired characteristic and the level of expression in strains containing wild genetic background.
- Capability of GMMs containing local wild genetic constitution to host and transmit local pathogen isolates.
- For drive systems, the rate of spread of a transgene in cage populations containing wild mosquito isolates and compared with Phase 1 predictions.
- For population suppression strategies, the rate of suppression against wild mosquitoes in cage trials.
- Egg hatching rates in crosses to wild mosquitoes.
- GMM release simulations in large outdoor cages.

Entomological activities in ecological confinement

- Establishment of go and no-go criteria.
- Compatibility with other mosquito control measures.
- Measures of GMM dispersal.
- Baseline studies of vector composition and abundance.
- For drive systems, the rate of spread of a transgene in wild populations and comparison with predictions from Phase 1 and Phase 2 physical confinement.
- Measures of transgene functionality and mutation rate.
- For population suppression strategies, the rate of suppression against wild mosquitoes.

[23] "Wild" refers here to a colony of mosquitoes isolated recently from the target population or a sample actually collected from natural populations and used without colonization. Such colonies are genetically more similar to natural mosquitoes than highly inbred laboratory strains.

- Randomized treatments of similar trial sites.
- Model refinement based on Phase 2 entomology and epidemiology observations; estimation of impact on EIR.
- For population suppression strategies, refined measures of relationship between sterility and population suppression.

Epidemiological activities in ecological confinement

- Measures of the ability to sustain development of local pathogen isolates as an indication of potential for transmission.

Phase 3. Staged open-field releases

Phase 3 is likely to begin with limited releases intended to understand the delivery requirements and functionality of GMMs under different circumstances, such as different ecologies, mosquito demographics and seasons. Large trials to determine epidemiological impact should only be planned after this information is at hand, as it will be necessary for trial design and interpretation. It is recommended that randomized cluster trials be included in the design for late Phase 3.

Entomological activities

- Compatibility with other mosquito control measures.
- Direct measures of EIR when possible.
- Baseline studies of vector composition and abundance.
- For GMMs with drive systems, the rate of spread of a transgene in wild populations and comparison with Phase 1 and Phase 2 model predictions.
- Measures of transgene functionality, phenotypic stability and mutation rate.
- Measures of GMM dispersal.
- For population suppression strategies, the rate of suppression of wild populations.
- Model refinement and validation based on Phase 2 entomological and epidemiological observations.
- For refractory GMMs, measures of native pathogen development and transmission in progeny from natural matings of the GMMs to wild mosquitoes.
- Methods for measuring or estimating GMM frequency and cross-species gene transfer and consideration of how long these activities should continue (Section 3. Biosafety).

Epidemiological activities

- Disease incidence/prevalence studies during intervention trials.
- Post-treatment active and/or passive disease incidence/prevalence, and consideration of how long these activities should continue (Section 3. Biosafety).

Phase 4. Post-implementation surveillance

Like any public health intervention, GMMs will require ongoing monitoring to determine whether their efficacy has diminished with time or because of unexpected effects that become evident when

used in new areas. Appropriate measurement of the entomological outcomes that guided deployment of the GMM must be continued after the trials cease. Depending on the type of GMM technology and the deployment strategy, multi-year follow-up may be required.

GMMs that reach Phase 4 will have undergone extensive efficacy testing. Their behaviour in natural settings will be established by Phase 3 activities. However, it cannot be assumed that they will continue to behave as expected. By analogy with the implementation of insecticides used for long-lasting insecticide treated bed nets, indoor residual spraying and larviciding, efficacy can change due to changes in the genetic constitution of the mosquitoes or external factors such as weather and human activities. However, the intervention at this point is no longer experimental, but is a control measure whose ongoing effectiveness in a public health programme is being determined.

A subset of the epidemiological outcomes that were utilized during Phase 3 trials should be monitored in order to determine whether the positive effects on human populations are being sustained. It is likely that if the GMMs were deployed over large areas, only longitudinal passive clinical case surveillance would be practical. In case a loss of efficacy is noticed – similar to the appearance of insecticide resistance with conventional control – any second generation GMMs that may be created must also be tested in phases 1–3, and monitored in Phase 4.

Entomological activities

- Direct measures of EIR under novel conditions (when possible).
- For GMMs with drive systems, the rate of spread of a transgene in wild populations and comparison with model and Phase 3 predictions.
- Widespread intermittent sampling of transgene functionality and mutation rate.
- Wide-scale intermittent measurement of GMM dispersal and gene flow.
- For population suppression strategies, sampling of the degree of suppression of wild populations.
- Model refinement based on entomological and epidemiological observations.
- For refractory GMMs, observation of native pathogen development in mosquitoes collected in disparate settings.

Epidemiological activities

- Longitudinal passive case detection of targeted disease and other mosquito-borne diseases.

Capacity building as an essential component of control measure durability

Durable efforts to conduct trials and to implement successful GMM interventions require strong intellectual understanding, cultural intimacy and logistical capabilities in locations where technologies are being implemented. Given the breadth of activities that have been described above, these require personnel and laboratories prepared to perform regulatory, medical, epidemiological, social and entomological activities. Further sub-specializations will be required: medical entomology, molecular biology, statistics and diagnostic analysis to name a few. It is simply impossible for these capacities to be supplied without reliance upon well-trained national personnel.

During trial design, an explicit personnel plan for the project should include the specific types of supporting expertise that will be required and the degree to which the project can and must take advantage of national capacities. When specific abilities are lacking, a strategy for training national personnel to satisfy these needs should be planned and undertaken. Sufficient lead-time for training must be part of the trial design, and a commitment to retain trained personnel in the trial will be important to ensure continuity, and allow for deep understanding of and involvement in the project. These personnel will play vital roles not only in trial conduct, but also in regulatory interactions and long-term monitoring activities.

For many national staff, training opportunities will be professional highlights that may make them eligible for national positions of authority and responsibility. Therefore, with their knowledge of personnel, technologies, and national regulatory and political avenues, they constitute invaluable long-term national focal points for future potential novel interventions. Commitment to providing assistance for training lays a foundation for future strength and independence for national research activities.

Capacity includes facilities. Even though construction of major facilities will be beyond the resources of most trials, increases in the capacities of facilities can include provision of scientific equipment, computers and software required for the trials, as well as the necessary improvements in biosecurity to achieve risk mitigation goals. Some structures, such as entomological-contained trial facilities, will be so specialized that support for the construction will likely come from the trial programme or in combination with other studies that could capitalize on the existence of a multipurpose facility such as the "Malaria Spheres" in Kenya. These kinds of facilities can be used to perform studies on mosquito behaviour, life history and non-GMM interventions. Coordinating investment in their construction provides a long-term foundation for wider sustained trials of vector interventions and research activities.

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3. Biosafety

Summary: Biosafety in the development of GMMs focuses on reducing to acceptable levels any potential adverse risks to human health and the environment that might be posed by these technologies, keeping in mind the known adverse effects of vector-borne disease. Risk analysis contributes to the achievement of an appropriate level of safety. Risk analysis takes into account that an event may occur but it may or may not be harmful in particular circumstances. Upon evaluation, some risks may be judged as negligible. Moreover, effective RM can make many risks acceptable. Overall biosafety RA should determine: the potential hazards and the mechanisms of impact for GMMs on wild populations of target and non-target organisms; the likelihood and magnitude of any harmful impact on the receiving environment; and, the levels and consequences of uncertainty associated with these effects. RM should provide appropriate measures to mitigate harm or uncertainty associated with changes to target organism populations or the wider receiving environment. Thus, RA allows researchers and regulators to determine the appropriate types and levels of GMM testing that will contribute to effective RM. Risk communication ensures that there is a well-documented explanation of what risks have been identified, how they have been assessed, what the acceptable level of risk is, and how RM may be able to achieve acceptable levels of risk.

The development and testing pathway for GMMs should be phased, with RM measures proportionate to the level of risk to humans and the environment at each phase. For example, confinement in early phase trials mitigates concern about long-term or large-scale spread and provides an opportunity to assess the likelihood and impact of hazards for which little or no empirical data exist at that stage. As more information becomes available, later stages of testing may need a less precautionary approach.

Studies in Phase 1 can provide data on risks that can be addressed by observing changes in behaviour and ecologically relevant characteristics of mosquito populations in small-scale laboratory experiments. With respect to biosafety testing, this Phase primarily focuses on the relevant characteristics of the GMOs themselves, and on laboratory experiments that can assess pathways that might lead to harm. In Phase 2, RA data are obtained in trials conducted under physically or ecologically confined conditions. This phase gathers RA data to reduce uncertainty regarding effects identified in Phase 1 and allows assessment of health and ecological effects under more realistic levels of exposure. Staged open field trials under Phase 3 can gather data under even more realistic conditions and using less confined measures than in the previous phases. In preparation for Phase 4, RA should include issues such as the potential for the movement of GMMs beyond the boundaries of a release area and the evolution of resistance, and will determine the necessary scope of post-implementation monitoring and management. The choice of risk comparators changes in its emphasis as testing moves through the various phases. At each stage a range of comparators may be needed to evaluate risks and performance across different dimensions.

Risk analysis that focuses on the phenotype (rather than the individual molecular modifications) provides a robust and appropriate approach to the assessment of GMMs. Risk analysis for GMM should be embedded in a broader benefit-risk analysis before decisions are made on large-scale implementation for public health purposes.

Biosafety considerations for GMMs address their safe use through the proper assessment of risks to the environment and human health, and the proper management of those risks. Risk is the combination of the magnitude of the consequences of a hazard (an unwanted event), if it occurs, and the likelihood that the unwanted consequences will occur. Risk analysis is an objective process to identify what hazards are relevant, how significant the risks are, how they can be managed, and how both the risks and their management can be communicated effectively to all concerned. Risks should be examined and responded to through established protocols within a risk analysis framework determined by a national policy on environmental and human health risks, and their

acceptance or management (US-EPA, 1998; EFSA, 2006, 2013; CBD, 2012).[24,25] Risk analysis may also take into account other types of concerns in addition to those related to human health and the environment (such as social or economic hazards, or hazards that would jeopardize the successful completion of the trial), but this section only deals with biosafety concerns.

Various examples of risk analysis processes are available including: a broad international standard;[26] national environmental guidelines;[27] and GM biosafety and risk frameworks referred to above. Across this range of guidelines, risk assessment (RA) is defined as a methodological approach to define and characterize hazards, and to estimate the exposure or likelihood of each hazard occurring as well as the potential adverse impact of the hazard (harm). In a phased series of testing, specific hazards would be addressed at each relevant phase. RA includes identifying hazards (those for which some direct or relevant evidence has been demonstrated), weighing the strength of evidence for such hazards, characterizing the risk and developing risk management (RM) strategies (through procedures, guidelines and regulation) to accept, avoid or reduce risk. The RA and RM strategies developed during laboratory testing and pre-release confined studies of any GMMs need to address two concerns: the effects of an escape or accidental release on a receiving (open) environment; and the effects of testing or release on human health.

RM of GMMs should be proportionate to the likelihood and magnitude of any potential hazards for which there is evidence. In countries with defined environmental policies and protection goals, these national policies provide the framework for determining acceptable risk levels. Observations of significant environmental effects at the various stages of GMM trials and implementation do not in themselves demonstrate a risk unless the outcomes are harmful. The impact of the effects must be evaluated, and the acceptability of risk is a policy decision that reflects the overall impact. During testing of phases 1–3 for GMMs, biosafety is the main decision-making determinant related to risk, but at the operational stage (Phase 4) decisions would also consider benefits and costs (including RM measures and any unmanaged residual risks). It is essential that potential risks be assessed and managed to ensure that modified mosquitoes are not more detrimental to human health (by increasing disease burden or severity) or to wider biodiversity (by adversely altering ecosystem structure and function). A reasonable overall standard in a RA would be whether a specific GMM implementation "causes more harm" than populations of wild mosquitoes managed under current practice, as has been used in Australia (Murphy et al., 2010). This standard is defined by specific endpoints that address harm to human health and particular qualities of the environment, and the elaboration of these endpoints would be the basis for studies to gather data to enable a RA to be conducted. At each level, risks specific to the genetic modification should be distinguished clearly

[24] The Cartagena Protocol on Biosafety to the Convention on Biological Diversity: http://bch.cbd.int/protocol/, accessed 25 May 2014.
[25] Australian Office of the Gene Technology Regulator. Risk analysis framework:http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/content/riskassessments-1, accessed 25 May 2014.
[26] ISO 31000:2009 risk management – principles and guidelines: http://www.iso.org/iso/news.htm?refid=Ref1266, accessed 25 May 2014.
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from those generic risks associated with the release of conventional laboratory or factory-reared insects.

The earlier development of GM technologies, principally for plants, provides a baseline for comparison of the differences between GMMs and wild-type mosquitoes that might result in environmental risk posed by the former. ERAs for GM plants are mandated for national regulatory agencies in many countries, for example, by the European Food Safety Authority (EFSA, 2010). These regulations follow a standard procedure to assess the risk of the technology to the environment (as set out in the CPB),[24] as well as to human health. Principally, this involves assessing the characteristics of the modification at the molecular, ecological and environmental scale, taking account of appropriate scientific evidence and uncertainty. While some of the goals and specific details will differ (such as the intended purpose of managed GMM release in alleviating disease burden, and the mobility of mosquitoes), the basis of biosafety guidance for GMMs will be built and adapted from existing frameworks for GM plants. Other useful precedents are provided from experience with biological control agents and GM vaccines. Each of these technologies exhibits unique features, but it is important that risk analysis frameworks are consistent wherever possible.

3.1 Considerations for risk analysis

Risk analysis is described in terms of risk concern, RA, RM and risk communication. Risk concern relates to awareness about issues related to both technology and social values, and in each case needs to be supported by evidence that demonstrates a concern has a plausible mechanism. RA and RM of GMMs require the development of risk frameworks in which scientific evidence is used to assess the probability that an adverse event (a hazard) will occur and the extent of harmful consequences associated, with and without mitigation.

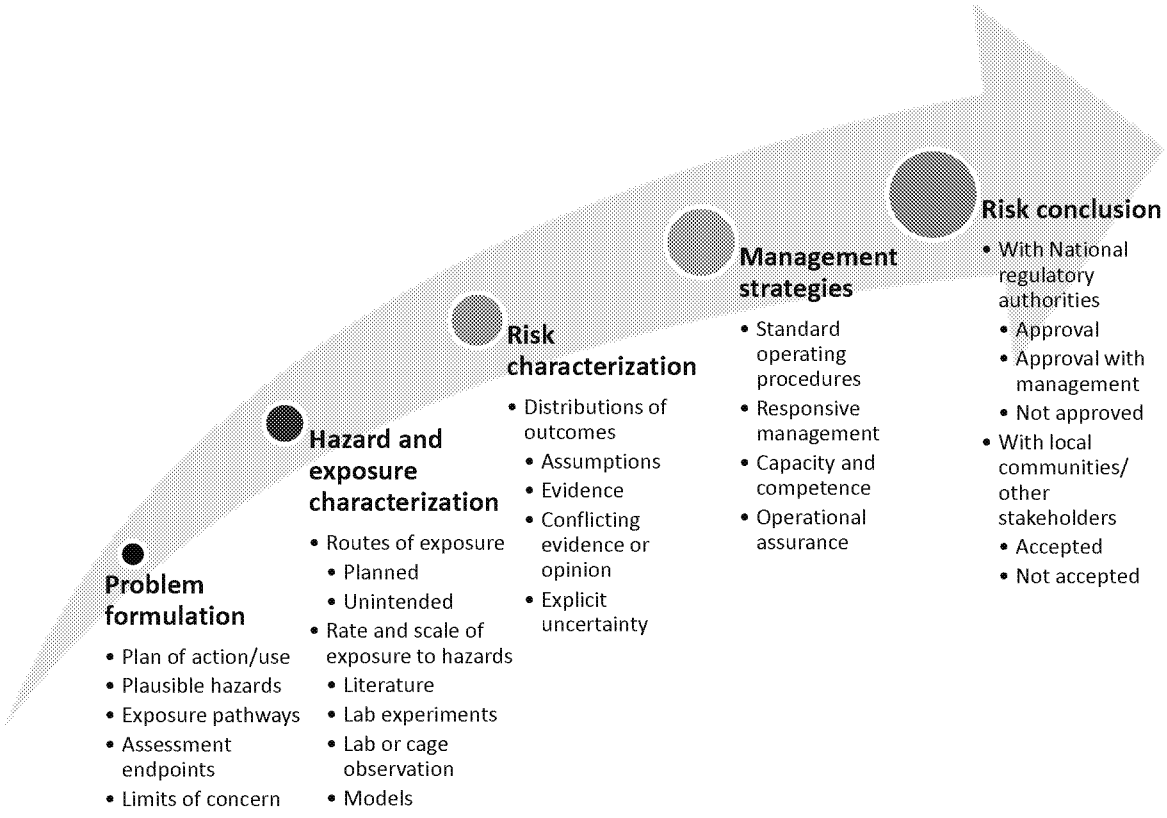
Both quantitative and qualitative risk analyses may be considered for GMMs. Quantitative risk analysis attempts to assign numeric values for the probabilities of various adverse events and to the assessment of the potential loss. Qualitative risk analysis assigns categories of risks, sometimes with relative scores reflecting the range of outcomes. Quantitative frameworks allow the expression of risk as probability distributions of adverse outcomes. Definitions and uncertainties in qualitative risk analysis can be expressed in scales that allow some approximate quantification (e.g. high, medium, low or negligible). Once risk is assessed, appropriate RM strategies can be devised and their efficacy also may be quantified in some cases. The wider environmental RA and RM guidelines referred to earlier from the United Kingdom[27] give useful guidance on how to assess the credibility or uncertainty of evidence in risk analysis, as does the Australian GM risk framework.[25] Quantitative risk analysis frameworks based on probabilistic and subjective estimates of social outcomes, such as that used for releases of *Wolbachia* infected mosquitoes in Australia (Murphy et al., 2010) may become useful in developing appropriate guidelines for the release of transgenic mosquitoes. This approach to belief networks can provide a robust quantitative framework for risk analysis that incorporates subjective evidence.

Risk analyses must be undertaken on a case-by-case basis to identify and manage any adverse effects to the environment and/or human health. The components of risk analysis have been described thoroughly in several venues, for example by Australia's Office of the Gene Technology

Regulator (OGTR),[25] the Convention on Biological Diversity (CBD, 2012), the EFSA (2006, 2013), United Kingdom's Department of Environment, Food and Rural Affairs (Defra)[27] and the USA's Environmental Protection Agency (EPA, 1998). Environmental risk assessment (ERA) for GMOs usually follows a multi-step process.

1. *Problem formulation*, which begins by considering concerns about risks arising from technical, social and other perspectives; it involves identifying the characteristics of the GM organism that might, on the basis of practical or theoretical evidence, cause harm to the environment and/or human health, and determining how this harm might manifest and what/who is at risk of this harm, along with an appropriate comparator for the risk.
2. *Hazard characterization*, determining the magnitude of the harm if it were to arise.
3. *Exposure characterization*, determining the likelihood of the hazard occurring.
4. *Risk characterization*, determining the level of risk, the product of the hazard and the exposure.
5. *Risk management*, selection of management strategies to alleviate/mitigate any identified unacceptable risks.
6. *Risk conclusion,* which is the outcome of the *risk evaluation* taking into account the residual risk remaining after feasible RM and the acceptability of that risk; it is important that the nature of the risk and its effective management or acceptance can be communicated effectively to those who have expressed risk concerns leading to the original problem formulation.

Figure 3.1 Example components of the RA process*



*Although portrayed as a linear process where knowledge and conclusiveness increase at each step, some of the components may flow in parallel or loop back to previous steps.

The problem formulation for an environmental risk begins with the planned actions for release of GMM and the identification of any potential hazards that may arise through plausible pathways. Examples of general hazards related to the release of GMMs include:

- release of the GMMs might increase transmission of the target or other diseases;
- release of the GMMs might cause a significant biting nuisance;
- release of the GMMs might result in disruption to valued ecosystem components.

However, it is important during the problem formulation process to identify any specific hazards of concern regarding the particular GMM technology being tested and/or the environment in which it will be tested. Harm may be specified in some national environmental regulation, for example, in terms of threats to particular endangered species or habitats.

An important concept of risk analysis is that while an event theoretically may occur, it will not necessarily be harmful, because either it does not have a perceived negative effect or it does not

have an effect specified as harmful in regulations. Many risks may be judged to be negligible, such as when the probability that the event will occur is extremely low or the potential harm resulting from the event is minimal. Even when potentially harmful events are identified, the practical level of risk to which the public is exposed in many cases can be reduced to acceptable levels by effective management.

3.2 Site characteristics

Baseline information on key ecological, environmental and site characteristics is important to ensure that field trials can be adequately planned and interpreted. Selection criteria might include the distribution of principal vectors in the release area, the location of mosquito larval sites, climatic conditions, knowledge of active transmission (if any) of the target disease pathogen at the site, geographical isolation of the site for confined trials so that there is a negligible chance of any impact outside the trial area, existing data on the transmission dynamics of the target disease, existing surveillance and control systems for both vectors and disease, the likelihood of obtaining regulatory, social and political approval for research on GMMs in the study community and surrounding areas (Sections 4. Ethics and public engagement and Section 5. Regulatory frameworks), and the ability to continue existing vector control practices.

3.3 Appropriate comparators

The choice of non-modified mosquito comparators will be essential in RA of any hazards associated with the transgenic modification. In some phases, such as in Phase 1, the ancestral laboratory line from which the transgenic mosquito line was derived is a logical comparator. A potential benefit for this as a comparator is that genetic similarity could be maintained allowing precise scrutiny of the molecular modification in terms of genetic and phenotypic viability and variability. A disadvantage of using ancestral laboratory lines is that the loss of fitness (due to intensive rearing in the laboratory) may lead to a less precise RA relevant to the characterization of the genetic modification compared to wild populations. Choice of alternative non-modified comparators (such as field-derived strains of the modified species) will require careful scrutiny of the genetic background together with physiological and behavioural characteristics. Such comparators may be more appropriate for field comparisons in later stages. For example, under self-limiting approaches, mosquitoes sterilized through more conventional irradiation methods may provide an appropriate counterpart for RA. Defining clear points for comparison, for example, a phenotypic characteristic such as adult longevity, will ensure that the risk evaluation remains credible, proportionate and focused.

The comparator for GMM in field trial phases would be the wild-type mosquito in that location, and the comparisons at this stage relate specifically to the types of mosquitoes. However, at a field implementation scale, the novel mosquito control system incorporating GMMs may be compared with a conventional control system. The comparison is related to the scale and purpose at this phase and addresses the risks arising across the integrated systems of control.

3.4 GMM characterization

The parental background of the GMMs should be described, including the species and strain, the geographical source, the number of generations rearing colonies have been maintained and the extent of replenishment with wild stock. These characteristics permit an assessment of the differences, and their potential effects, between the GMM and the wild-type comparator. The genetic modification should be described, including molecular characterization, insertion sequences and location. The stability of the transgene is an important issue in determining if the characterization of the GMM remains valid over successive generations, which may be an important objective of Phase 1 laboratory studies.

In RA, statements on the modification undertaken, its original derivation and the effect it confers should be stated clearly. The methods used to generate the GMM lines and the sequences, genomic locations and schematic maps may be required. Information on the flanking sequences may be required to identify whether new open-reading frames are generated from an insertion. Original sources of vectors used for the molecular transformations, the source of donor genetic material, its size and intended function should be described. Information on the actual sequences inserted (or deleted), the size and copy number of detectable inserts, and the functional organization of the genetic material is necessary core information on the transgene. Details should be provided on the developmental expression of the transgene insert (or modification through knockout deletion based on transgenic technologies) during the life cycle of the mosquito. The RA should account thoroughly for the molecular characterization and consider the risk associated with the incorporation of molecular constructs or insertion mechanisms (for example, plasmids and transposable elements) into the modified mosquito.

A further aspect of characterization is the description of the GMM use or application. This should include an indication of the expected release rates, duration and spatial distribution of the GMM, along with any other measures that may be taken as part of the integrated control system (for example, the suppression of wild populations with insecticides before GMM release).

3.5 Hazard characterization

Hazard characterization will normally be specific to a particular GMM technology and scope of use, but it is possible to describe some of the more general possibilities that should be considered. A hazard may derive either directly from the intended effect of a genetic modification or indirectly through an unintended deviation from that intended effect. For example, the breakdown of the molecular function could lead to a loss of GMM efficacy and to potential changes in the impact on the environment and/or human health. To assess this, under both self-limiting and self-sustaining approaches, the RA should be associated primarily with the genetic modification.

Alterations to the biological characteristics of the GMMs may lead to new interactions with target mosquito populations. Examples of such potentially harmful alterations could include altered larval competition and accelerated maturation. Biological alterations such as those leading to increased insecticide resistance or human feeding might change vectorial capacity relative to wild-type populations. To predict the effects of a particular GMM release on the target population, it is

essential that appropriate phenotypic, behavioural and population level characteristics of the modified mosquito be assessed through laboratory experiments and trials. Although Table 3.1 provides a set of characteristics that are likely to be important in the understanding of the impact of a GMM on the target population, the most important and relevant characteristics should be identified and assessed on a case-by-case basis. This will ensure that appropriate RA criteria are established and thorough RM strategies are in place.

Under self-sustaining approaches, molecular characterizations must show that the transgene is sufficiently effective and the molecular construct linking effector transgenes to a drive system is sufficiently robust to ensure that the release of the GMM results in introgression of the genes into wild mosquito populations (James, 2005). Appropriate drive systems are crucial to ensure that a faster rate of spread of the genetic construct occurs than would be expected under standard Mendelian inheritance (Burt & Trivers, 2006). It is important to understand the essential aspects of the population genetics of the transgenic modification as some gene drive systems might be expected to cycle to and from fixation in populations. Similarly, molecular characterizations for self-limiting approaches need to consider the expression patterns of the effector gene, including whether expression is under appropriate gene control and stable within the genome. The RA for GMM should consider the stability and specificity, in relation to the intended effect, of the transgenic material at the population level and the consequences of incomplete or partial transgene function.

Identifying the risks associated with incomplete transgene function in individual mosquitoes will have implications at different phases of testing, including at the population level and in confined field trials. Reduced penetrance (the proportion of a given genotype that expresses the phenotype) of a transgene in a population may pose a risk to the receiving environment and/or human health if it affects the ability to detect transgenic individuals or reduces the anticipated control benefit. For self-limiting strategies, low penetrance at the population level will affect efficacy, and population trials should aim to quantify any human health risk associated with this in a disease vector control system, for example, if the capacity for pathogen transmission is not sufficiently blocked. Such risks might be managed with core quality control measures (such as genetic markers). With self-sustaining strategies, incomplete penetrance of a transgene may not influence the outcome of long-term control but might affect the initial success/spread of the transgene. Methods must be provided to allow for discrimination of GMM within the environment and to monitor the maintenance of transgene integrity. This will also be important for assessing GMM efficacy in later phases of testing.

Further possible hazards could arise from random integration of the effector gene, such as low efficiency and position effects on transgene expression and the potential for insertional mutagenesis. It is likely that transgenic strains exhibiting these effects would not be considered suitable for eventual deployment. It is expected, therefore, that most of the potential hazards resulting from random integrations would be eliminated during the product development process. Specific strategies to reduce random integration might be employed. An example of such a strategy is provided by the two-tiered approach to the molecular modification of mosquitoes, which in the first stage involves inserting a target at a suitable chromosomal site, and in the second involves recombining the effector gene into the target site (Nimmo et al., 2006; Sethuraman et al., 2007; Isaacs et al., 2012).

It is conceivable that multiple transgenes might be used to achieve the desired effects. Synergistic genetic interactions and unexpected phenotypic consequences of multiple genes should be assessed to determine if they pose a potential risk to the receiving environment, and thus require RM strategies. It is important to consider how to approach the RA and RM of 'stacked' events (multiple transgenic modifications) to ensure the efficacy of these transgenic modifications and manage any risk associated with the evolution of resistance. Characterization of stacked events should consider the stability of the inserts, the expression of the events and potential synergistic or antagonistic effects arising from the combination of the transgenic modification and the phenotypic characterization of the effects through life-table, behavioural, and/or population observations/experiments. Appropriate comparators for laboratory studies might include the conventional parental strains or the equivalent wild mosquitoes, the lower stacked event lines (provided appropriate RA/RM advice exists) and wild-type mosquitoes. Characteristics based on the phenotype (rather than the individual modifications), and their interpretation from available baseline data, provide a robust and appropriate alternative to the full RA on every individual molecular modification in a stacked GMM. Therefore, RA should assess the impact of GMM in terms of phenotypes rather than individual modifications in stacked, multiple transgenic modifications.

Some interactions of GMMs with other organisms in the environment may result in hazards and may therefore pose risks to the receiving environment. As mentioned above, hazards might include undesirable changes in populations of interacting organisms, physiological or behavioural differences in the GMMs that affect nuisance impacts, or increased opportunities for transmission of non-target diseases. Preliminary ecological or behavioural patterns associated with modification related to such potential hazards should be assessed through longitudinal, population-level cage trials of both GMM and non-modified comparators over time scales relevant to the patterns being observed. The use of semi-artificial microcosm and mesocosm systems (Lawton, 1995) in trials that aim to mimic the key aspects of the receiving environment would allow the population dynamics and population-level characteristics of the GMM to be characterized more accurately than simple laboratory population cage studies. These small-scale laboratory or caged environments attempt to provide potential for interactions with a limited range of ecological complexity, which would provide a bridge into more comprehensive physically and/or ecologically confined field trials. Careful choice of experimental design and planning may allow a range of potential ecological characterizations, which might include those below.

- The role of density dependence in the population dynamics. The timing of density-driven events that affect survival, development rate and/or fecundity can be explored using population cage and semi-artificial microcosm and mesocosm trials, appropriate statistical analysis and mathematical modelling.
- Comparison of discrete dynamics, for example, seasonal factors such as rainfall, versus continuous dynamics, such as competition for host finding, under semi-artificial conditions allows estimates of the effects of seasonal versus aseasonal effects to be discriminated.
- Exploring preliminary release numbers/schemes (for self-limiting approaches) or invasion potential (for self-sustaining approaches) of transgenic lines.

Novel interactions of the GMMs with non-target organisms (NTOs) could have important consequences for ecosystem function and services (EFSA, 2010). An example might be if the

abundance of the NTO species was reduced and was an important seasonal part of a food web for predators. The direct exposure of non-target species to the GMMs, or to transgene products, requires careful assessment in order to identify risks and, if they exist, manage and mitigate them. Population-level microcosm or mesocosm trials could evaluate the specific effects of the GMMs on NTOs, where these have been identified. The choice of appropriate NTOs (such as predators or competitors, decomposers) is a complex decision but could allow the preliminary effects of particular high-value inter-specific and trophic effects to be evaluated. The EFSA's guidance (2013) for the choice of NTO in the environmental RA of GM insects suggests that these should include natural enemies, competitors, pollinators, species of conservation, cultural or food chain value, decomposers and host animals. Similar approaches might translate to appropriate choice of NTO in small population-level studies with GMMs. With appropriate controls (with/without competitors/natural enemies/decomposers) the preliminary criteria of the RA on NTOs can be established.

An alternative scenario that has been proposed for GMM population suppression approaches is the possibility that a resulting empty ecological niche may be filled by alternative unwanted species. For example, laboratory studies of competitive interactions on (non-modified) *Aedes aegypti* and *Aedes albopictus* demonstrate that *A. albopictus* larvae are superior competitors for resources compared to *A. aegypti* over much of their range (Juliano, 1998; Daugherty, Alto & Juliano, 2000). This has implications for the invasion and establishment of *A. albopictus* after suppression of *A. aegypti* to inhibit dengue transmission. Available information from laboratory and field ecological studies will help to assess the ecological and health implications of the empty niche hazard. For instance, in the case cited, available evidence indicates that *A. albopictus* plays a minor role in dengue transmission due in part to different host preferences and reduced vector competence (Lambrechts, Scott & Gubler, 2010).

There may be additional concerns about hazards related to possible direct human health effects arising from GMMs, such as nuisance biting or allergic reactions. In this regard, it is important to keep in mind that only female mosquitoes bite humans or animals. Nuisance biting would increase if female mosquito abundance increases, but would not be expected to pose a disease hazard with GMM applications intended to either reduce populations or replace wild populations with similar numbers of refractory mosquitoes. Increased allergenicity of GMMs has been proposed as a speculative risk to humans, though no supporting information is available. While ingestion has been suggested as a possible route of exposure, this is likely to be quite rare and thus unlikely to pose a significant hazard. The most likely route of exposure to GMMs is via biting. The saliva of all mosquitoes naturally stimulates an immunological response in most persons and a strong allergic response in some (Peng & Simons, 2007), and there is considerable cross-sensitivity to the salivary proteins from wild populations of mosquitoes. Therefore, determining a GMM-specific response in the context of such natural variability will be difficult. However, with GMM technologies in which female mosquitoes will be released or transgenes will be expressed by female progeny, it is appropriate for an RA to consider whether a transgene product is expressed in the saliva and, if so, whether this protein is significantly similar to a recognized allergen. In such a case, further studies may be warranted and established; validated protocols for assessing allergenicity of proteins by dermal exposure should be followed.

The efficiency of quality control for effective management of the modification of mosquitoes, such as the operational ability to derive only certain types (for example, one sex in male-only releases) of transgenic insects for release, may be relevant to an RA. The methods and degree of separation necessary depend on the scale of the trial or planned release and the GMM technology under consideration. Achieving the desired sex ratio and levels of separation require appropriate operational protocols. In laboratory trials and population cage experiments, the ability to discriminate and separate relevant strains of transgenic mosquitoes should be evaluated. RM options should focus on how necessary it is to obtain absolute (100%) separation in order to achieve safety and efficacy endpoints in the trial/release. Control may be achieved even when some females, which do not contribute to control in sterile male release programmes, are released. For example, in the use of a conventional radiation SIT method, the local elimination of *An. albimanus* in El Salvador was achieved with the release of sterile insects of which approximately 14% were females (Lofgren et al., 1974). The quality and numbers of released GMM needed to achieve intended vector or disease outcomes should be specified and explained in the release plan. The risks arising from not achieving that level of quality or numbers in releases should be assessed and managed.

3.6 Utility of mathematical modelling for RA

RA can be enhanced by coupling experiments and/or observations with mathematical modelling. Mathematical modelling can highlight the range of parameters necessary for RA. The overall aim of mathematical modelling within the RA context is to predict behaviour based on properties and assumptions of transgenic modification that may be helpful in assessing the likelihood of events. For example, given a specific set of molecular modifications, mathematical models might be used to predict whether or not the fitness of the GM mosquito will be enhanced by the molecular modification (Box 3.1).

Box 3.1 Modelling to determine the net effect of altered fitness

In a model system where GMM containing a particular anti-pathogen effector gene were continually fed on mice with a high level of parasites, increased fitness of the malaria-resistant mosquitoes was observed (Marelli, Rasgon & Jacobs-Lorena 2007; Smith et al., 2013). Given such an observation, modelling might be used to determine the net effect of increased fitness, the expected frequency of infected mosquitoes and possible effects on transmission. The appropriate theoretical framework to undertake this RA would be a full analysis of the life history combined with competition experiments. Essentially, this consists of determining both aspects of fitness associated with survival and aspects of fitness associated with fecundity and reproductive success (Stearns, 1992; Roff, 2002; Godfray, 2013). This could involve laboratory studies that focus on a selected set of core parameters (Table 3.1) associated with the specific genetic modification coupling life-table experiments, experiments on small batches of modified and non-modified mosquitoes (such as split by age, sex or strain) in cohort experiments, and mathematical modelling.

Mathematical modelling of inter-specific interactions might be useful to reveal potential structural alteration to the ecological (biotic) effects. For example, self-limiting strategies where population suppression is the goal are expected to lead to non-uniform competitive effects, as population interaction strengths with other species will differ at high and low densities. Under self-sustaining strategies, assessing whether the heritable modification will have an impact on the ecological

competitive ability of the GMM and/or ecological interactions could be accomplished using data from small-scale semi-artificial population trials in the laboratory.

3.7 RA and RM considerations at different testing phases

As explained above, given the various potential hazards that might be enumerated, RA and RM must be focuson the particular GMM application under examination and its objectives within the phase of testing under evaluation. Specific RA and RM considerations will differ between various GMM technologies and in different phases of testing. For example, the level of exposure will be less in contained trials than open releases, and with sterile GMMs versus those that are self-sustaining. At each level of testing, from laboratory through to field trials, the aim of specific RA and RM approaches should be to ensure safety and to quantify or provide a qualitative rank of risks associated with the eventual deployment of the GMMs.

Transition from each phase of testing to the next should involve both a retrospective validation of the RA/RM that was put in place at the beginning of the phase and an evaluation of whether the performance characteristics that were measured warrant progressing to larger trials according to previously designated efficacy and safety endpoints. In addition, any hazards that were unforeseen before starting the previous phase should be considered in the decision along with additional management measures. The decision to move forward with further testing will require approval from the appropriate oversight and regulatory bodies at each phase (Section 5. Regulatory frameworks).

3.7.1 Phase 1 – Laboratory studies including Laboratory Population Cages

RA for Phase 1

Phase 1 testing will be conducted in a laboratory or insectary under physically confined conditions. Because this is an early stage of development, there will inevitably be limited information on the stability and effect of genetic modifications and a cautious approach is essential, primarily due to uncertainty rather than any established hazard. RA in preparation for Phase 1 will determine the conditions under which laboratory studies can be conducted, including the acceptable level of exposure to GMMs by research personnel, acceptable security measures to prevent GMMs from escaping, and appropriate methods for disposing of waste materials.

Risk management

RM measures for environmental impact will include appropriate containment[11] of live mosquitoes and destruction of dead mosquitoes and waste materials (if there is evidence that these may be a hazard) (Benedict, Tabachnyk & Higgs, 2003). RM measures for human health would include ensuring GMM colonies and feed sources are free of human pathogens, ensuring laboratory staff are not carrying mosquito-transmissible diseases, and limiting unintended biting opportunities (to guard against disease transmission) by preventing and removing mosquitoes flying outside cages and by ensuring that laboratory staff wear suitable protective clothing. RM to respond to escapes from the laboratory would include escape detection systems and standby mosquito control capacity sufficient to control adults within the dispersal range of the mosquitoes and/or conducting experiments in

seasons when adult dispersion and mosquito larval sites will be limited. Where testing of disease transmission or infection cycles in GMMs is undertaken, particular care should be taken to ensure the safety of laboratory staff. All of the above are also good practices in rearing non-GM mosquitoes, particularly when they are being handled in areas where they are exotic and could establish following escape, and build upon standard precautions.

Studies to gather data for deployment RA

This early phase of the development of a transgenic mosquito focuses primarily on the biology of the target species and integrates molecular, genotypic, phenotypic, behavioural and population-level characteristics (Section 2. Efficacy evaluation). The data collected at this phase to address identified risks will focus primarily on the genetic modification of the mosquito and its interaction with and distinctions from the comparator mosquitoes in the laboratory. Alterations to target populations through changes in the demographic size, structure or behaviour may have a detrimental impact on the wider environment and/or human health. Experiments to determine whether these alterations may lead to specific harms can begin to be addressed at this stage. Examples of Phase 1 studies that characterize those aspects of the biology of the modified mosquitoes and inform the RA associated with the eventual deployment of GMMs have been previously described (Benedict et al., 2008) and are additionally detailed in Table 3.1.

Results of Phase 1 testing will determine whether trials may proceed safely to Phase 2 ecologically confined trials or whether physical confinement is a necessary intermediate step to obtain additional safety information.

3.7.2 Phase 2 – physically and/or ecologically confined field trials

RA for Phase 2

Physically confined (contained) and ecologically confined field trials conducted under Phase 2 allow data to be collected that require a larger scale or more natural conditions in order to be detected. RA will determine the level of confinement required in Phase 2. For some GMM technologies, it may be decided that physical confinement is not a necessary step in the testing pathway and that conditions of genetic or ecological confinement allow for sufficient risk reduction. For example, a regional standard in North America accepts biological confinement for sterile transgenic arthropods, provided there is data on the efficacy of sterility (NAPPO, 2007). Physical confinement may be less important in cases where Phase 1 results have demonstrated that there is limited potential for dispersal, for example, for trials where the GMM's progeny do not mature to adults, or where the GMM is not expected to persist (for example, transgenically marked laboratory strains with intrinsically low fitness in the wild). Previous evidence from laboratory or other confined trials may demonstrate that protocols to discriminate the sex of the released mosquitoes, and their phenotypic properties, are sufficient to ensure safety in an ecologically confined trial. Regulatory requirements will likely differ for physically confined versus ecologically confined trials.

Understanding the risk associated with a breach of physical/ecological confinement requires appropriate consideration. A breach of physical confinement may lead to the loss of transgenic mosquitoes or loss of genetic material into the wider receiving environment. Breaches of physical

confinement might be classified in terms of the potential magnitude and type (Benedict et al., 2008). Breaches might be caused through natural disasters, structural failures, human error/accidents or deliberate actions. The RA should take into account cage designs, experimental planning, emergency preparation, training, and site security.

RA should ensure that a mechanism for practical and reliable discrimination of GMMs and wild mosquitoes is available (for example, through the use of fluorescent dyes or dusts and/or phenotypic or genetic markers). Where release of male-only GMMs is part of the system, methods for reliable sex-selection prior to release will be necessary to ensure an acceptable sex ratio is achieved. Other biological considerations for RA in preparation for Phase 2 testing would include what is known about the local dispersal and gene flow patterns for target mosquitoes and what pathogens they transmit in the receiving environment (Benedict et al., 2008).

Risk management

In confined field trials, risk will extend to greater varieties of environmental and target species effects. Risk associated with these trials must be managed by limiting the spatial and/or temporal scale of the planned release activity. Documenting the hazard/differences associated with the escape of self-limiting or self-sustaining transgenic lines through breaches will be an essential aspect to RM, including the containment requirements for cage design. It is anticipated that the risk will be lower with self-limiting GMM due to their lack of potential for persistence in the environment.

Physically confined field trials should give particular attention to cage designs and local environmental conditions at the chosen field site. Aspects of local geological, ecological and regulatory criteria will underpin the design of physically confined field cages and trial implementation (Facchinelli et al., 2011; Ritchie et al., 2011). Ecologically confined field trials may take place in locations that do not favour the long-term survival of the GMMs, or in ecologically isolated locations (such as an area surrounded by water, deserts or mountains). Combinations of physically and ecologically confined trials are possible.

Further simple RM measures, including restricted access, clear and well managed SOPs and appropriate ethical/cultural considerations (Section 4. Ethics and public engagement) could all be used to mitigate hazards associated with confined trials. While clear research protocols would be necessary beginning at the Phase 1 laboratory population trials, SOPs become increasingly important as testing through the tiered phases moves forward. An SOP is a written plan describing the procedures to be carried out during the field trial evaluation of GMMs. For example, a SOP would document how transgenic material should be moved from the laboratory to the field prior to release, the protocols for ensuring site security and cage suitability (Benedict et al., 2008), criteria for release strategies, surveillance during the trial and the post-trial removal of material and cages. SOPs should describe the lines of responsibility and the RM strategies and options for the trial. Monitoring the performance of containment measures, such as physical integrity of screens, the operation of entryways and adherence to SOPs will minimize risk from unintended release.

RM should include the monitoring of GMM populations within the trial area to ensure that the technology is having the intended effect on the target population. Periodic sampling of the GMM population in the trial should be undertaken to determine the stability of the transgene and any

recognizable change in the genetics of the population that may affect the impact of the technology. Key interactions with other species in the trial, which might indicate wider environmental impacts, should also be monitored in order to identify and characterize any unexpected harmful effects, and identify representative "sentinel" species.

There should be sufficient monitoring for the detection of any GMMs that escape confinement and establish unintended self-replicating populations in the wild. Control capacity that is proportional to the risk should be maintained to ensure that escaped GMMs do not persist in the environment. Where practical, measures may need to be taken to limit the establishment of GMMs within the potential dispersal zones, such as controlling wild mosquitoes and limiting available larval breeding sites. Standby control measures should take into account any behavioural attributes of GMMs that may differ from wild mosquitoes. Monitoring and control capacity should continue after the trial is completed for a period sufficient to ensure that there is no unintended persistence of the GMM or manifestation of unintended effects (Benedict et al., 2008).

Plans would need to indicate how residual populations in cages would be eliminated after a trial; in the case that the risk is determined to be negligible, this might simply involve allowing the material to enter the decomposer food chain. However, if such residual material were identified to constitute a hazard, more aggressive RM of residual dead material would need to be considered.

Studies to gather data for deployment RA

Because of the higher degree of influence of the environment on these trials, and the more limited ability to control levels of exposure to some environmental stressors, a greater number of experimental replications may be needed for sufficient statistical power compared to Phase 1 laboratory studies. Phase 2 allows evidence on GMM performance to be gathered under more natural conditions to provide an appropriate level of RA and RM before full implementation of open-field trials in Phase 3 (which are likely to be conducted in a location where the target disease is endemic). However, confinement in Phase 2 trials introduces differences from the natural environment that may affect the performance of GMMs and other organisms within the trial, so it will be important to be clear about the most relevant information needed to make decisions about moving forward.

Consideration should be given to whether the release of GMMs poses a risk through the persistence of functional genetic material within the GMM species and whether the transfer of the genetic material can occur between species. The transfer of stable genetic material from one organism to another without reproduction is called horizontal gene transfer (HGT). The risk posed by HGT from GM organisms is generally believed to be negligible (reviewed by Keese, 2008). No evidence of HGT from transgenic plants to microorganisms has been detected in the field over decades of observation and millions of hectares of planting (Keese, 2008), and occurrence of HGT from the relatively less abundant GMMs may be expected to be even more rare. Considerations relevant to RA for transgenic organisms, including GMMs, are whether the transgenes contain components that could plausibly confer a selective advantage to microorganisms with which the GMMs will interact, and whether acquisition of this trait would be harmful. RA would need to consider this on the basis of the known function of the transgene and whether that function is preserved in microorganisms.

Identification of clear endpoints to the Phase 2 field evaluation will require basic ecological, entomological and epidemiological information. Ecological processes such as density dependence and age structure affect the design of measures to mitigate risk to the wider environment, biodiversity and human health. This assessment should be considered in Phase 2. Density dependence is a process that leads to increased mortality, reduced development rate, and decreased fecundity or longevity as density increases. It is an important ecological process in the dynamics of most populations and evaluating its timing and effect in wild-type versus transgenic mosquito populations is of potential importance to the RA of modified mosquitoes (Yakob & Bonsall, 2009). The timing of important density-dependent processes with respect to the expression of the effector gene has substantive implications for the impact of some proposed genetic control suppression strategies. Under both self-limiting and self-sustaining approaches, timing the expression of the effector gene after the stage at which density-dependent effects are greatest (such as the larval stage of *Aedes aegypti* (Phuc et al., 2007; Legros et al., 2009) can lead to more effective suppression. Phase 2 trials should be structured to provide relevant information on the ecological processes critical to the evaluation, efficacy and success of the GMMs. Age structure can affect density dependence where different stages and ages within stages do not compete with each other.

Additional considerations for biological information to be acquired in Phase 2 testing will relate to the specific GMM approach under consideration. Suggestions for Phase 2 testing of mosquitoes containing a gene drive system have been described previously (Benedict et al., 2008).

3.7.3 Phase 3 – staged open-field releases

RA for Phase 3

The RA associated with site selection for open releases should consider the isolation of the site, the structure and knowledge of the vector population, the disease dynamics and the implications of any differential impacts among local communities. It should also consider the size of the open-field release site, which will dictate the site characteristics. When selecting the site, RA could make use of the substantial advances in technology and knowledge of geographical surveys (e.g. global positioning systems, geographical information systems and high resolution satellite images), and predictive models of habitat suitability. These methodological advances allow thorough analysis of temporally and spatially referenced data relevant to both mosquito ecology (Thomson & Connor, 2000; Malcolm et al., 2009) and disease burden (Gething et al., 2010).

Choice of appropriate site size and layout (randomized block, Latin square, Cox & Reid, 2000) will enhance both the biological and statistical validity of the open-field release. Cluster size and number should be predicated on the focused aims and endpoints of the staged open release (Section 2. Efficacy evaluation). Plans for open-field releases to assess efficacy of spread (e.g. competitiveness, longevity, dispersal) should consider the need for well-designed and replicated experiments at a spatial scale that limits the effects of immigration and other spatially dynamic processes. Similarly, RA and RM for open releases designed to demonstrate suppression and replacement potential should consider the measurable parameters (such as population density or the proportion of a genotype in the field population) needed to demonstrate conclusively the aim of the release. If the endpoints are focused on disease control then appropriate knowledge of the size of the human

population, level of disease burden and ethical issues related to testing of disease interventions (Section 4. Ethics and public engagement) should be incorporated into the RA. The evaluation of GMM effects on the incidence of the target infection will be part of efficacy testing (Section 2. Efficacy evaluation), but based on studies of vector capacity in phases 1 and 2, consideration should be given to the need for monitoring other vector-borne diseases.

The spatial scale of a proposed field trial may have environmental consequences through NTO effects within or outside the planned boundaries of the trial site. Risks associated with potential transgenic releases should consider the spatial pattern and the scale of the entomological/ecological risk (Getis et al., 2003). The effects of modified mosquitoes may extend to neighbouring areas if migration between populations can occur (Yakob et al., 2008). Determining the appropriate scale for a release strategy and the implications for adjacent non-target regions requires an appreciation of the relationship between ecological processes such as the timing of density dependence, demographic processes (Table 3.1) and spatial aspects (Lee et al., 2013). This can only be evaluated realistically during field trials. Assessing the different types of release strategy for both self-limiting and self-sustaining approaches is important, as knowledge of the connectivity between the population within the target zone and the surrounding populations is important in preventing any adverse increase in the entomological or epidemiological burden associated with the target mosquito.

Unintended transboundary movement becomes a potential risk with field testing and release. This could occur through natural dispersal or through human-assisted movement, either accidentally or through deliberate unauthorized transfer. Natural dispersal is a slow process for most species of mosquitoes, which normally remain within a few hundred meters over their life, unless transported by man or strong winds (Service, 1997; Getis et al., 2003). Areas that are unsuitable for host finding or breeding often further limit movement. Natural movement over substantial distances, including transboundary movement, would normally take many generations, which would be a far more likely occurrence of expanding self-sustaining populations. The proximity to borders, geographical barriers, prevailing winds and water flows, and vehicle traffic would affect the likelihood of transboundary movement. The presence of suitable habitats and hosts, and vulnerable ecological and social systems across a border where GMM might move would increase the potential for establishment and impact.

Risk management

RM in Phase 3 will be similar to Phase 2 above but will need to be expanded in scale to account for the lack of confinement. The evaluation of surveillance data would benefit from the availability of appropriate baselines before release (such as the level and seasonal pattern of disease burden, the past levels of the vector population, effects of conventional vector-control methods). A recall or control plan of sufficient scale to limit spread should be agreed upon and be available before field release, if there is ongoing concern about risk. At a minimum, an additional risk RM measure would be to stop GMM releases in the event that monitoring detects that an otherwise unmanageable and unacceptable hazard has developed. In such a case, a more extensive and intensive conventional control capacity may be required to eliminate any residual population of GMMs after release and dispersal.

There should be a procedure to monitor any degradation of efficacy in the GMM control system that may indicate that resistance to the effector has developed. The degree of resistance, its rate of increase and possible attendant hazards must be evaluated. Regular sampling of wild populations should be considered as a method to detect resistance.

Management should be put in place to avoid and detect transboundary movement in case neighbouring countries have not approved release for testing (Section 5. Regulatory frameworks). Field testing should be carried out at some distance from borders to avoid natural wind and water flows to other countries. Released GMMs should carry markers that ensure discrimination from wild mosquitoes. Monitoring between a release site and a border could indicate if there is any movement. In small trials, a treated barrier area downwind may reduce the chance of successful movement towards a border. Staff working on field-testing sites should be trained about the risks of moving living specimens and should observe transport protocols when moving any material. Post-trial monitoring should take into account the numbers of GMMs released, with the aim of achieving an appropriate level of sampling efficiency.

Studies to gather data for deployment RA

Phase 3, which is likely to involve a series of open trials of increasing size, duration and complexity, should provide the safety data that will be factored into decisions about the broad-scale implementation of the GMM technology. Open testing in Phase 3 will introduce opportunities to gather data on potential hazards in the risk analysis (Table 3.2) where these data can only be acquired under more natural conditions. It also provides an opportunity to evaluate the performance of GMMs integrated within complementary conventional control actions. However, considerations of environmental variability, reduced control of experimental variables, and the impact of these on proper experimental design and statistical power are even more influential at this stage. RA under field trials may provide information on whether the transgenic modification has any chance to increase vectorial capacity (the efficiency of vector-borne disease transmission) or vector competence (the capability of a vector to support the development of a pathogen) under particular circumstances (Table 3.1). Monitoring for changes in the incidence of the target infection or disease is addressed in Section 2. Efficacy evaluation. A failure to decrease vectorial capacity under self-sustaining approaches may result from a decoupling of the effector gene from the drive system. Vectorial capacity under self-limiting approaches is also associated with the quality control of transgenic releases. For example, incomplete penetrance of the modification may influence both vector capacity and potential disease burden. Phase 3 also may provide an opportunity to detect whether changes in the pathogen develop that decrease the efficacy of GMMs, an effect that may be difficult to determine in short-term trials.

Understanding endpoints and intended consequences of GMMs necessitates understanding the relevant aspects of mosquito biology and ecology. Basic ecological knowledge of mosquito vectors in receiving environments must be available to evaluate the benefits of transgenic mosquito releases and should be part of the overall research plan. For example, while population genetic studies on mosquitoes are common (Touré et al., 1994), at the time of writing, there have been practically no ecological studies of the effects of seasonality in West Africa on *An. gambiae* in relation to the forms that are present and how they are distributed in space; basic information such as whether *An.*

gambiae is resident in or repopulates disease-endemic areas remains unclear. The ecological difference between intrinsic population growth and immigration is substantial and requires assessment in order to validate risk estimates, define RM and determine appropriate endpoints. While extensive information on direct and indirect interactions through purpose-designed experiments would be desirable in any ecological field study (Bender, Case & Gilpin, 1984), key information for the RA of undertaking transgenic releases under open-field conditions should be proportionate and focused, requiring the development of sampling programmes (Silver, 2008). The impacts on human health and the wider receiving environment cannot be evaluated appropriately without this assessment.

Assessment of wild-type mosquito population size and dynamics is essential for both self-limiting and self-sustaining approaches. Mark-release-recapture measurements of wild-type mosquitoes can provide a baseline for assessing the necessary release ratio and the risks associated with releasing large numbers of transgenic mosquitoes. Assessment of population size, age structure and/or sex ratio post release should take into account sufficient time for a new equilibrium to be established. The fitness of a population should be assessed to determine if there is a risk of population increase in the longer term.

At the end of Phase 3, the GMM stands on the verge of routine use as a public health intervention. Sufficient data should have been collected to understand the effects of the GMM on disease transmission, ecological interactions and the spatial characteristics of dispersal and transgene persistence. This will have involved extensive post-release monitoring of wild populations for the transgene, widespread assays of the GMM for phenotypic and marker stability, and an assessment of the performance of the RA and RM strategies. These considerations will compose an important part of any decision to move forward with deployment, a decision that will necessarily also take into account broader cost-benefit, acceptance and national public health goals.

3.7.4 Phase 4 – post implementation

National regulatory authorities will take the results of risk analysis at this stage into account when making decisions about whether and how to allow large scale GMM deployment in their countries. National public health agencies would also consider the results of risk analysis in deciding whether to adopt GMMs as a component of their national disease-control programmes. The evaluation of risk, in the context of implementation, should be set against the benefits of GMMs in improving human health. Benefit-cost analyses provide the framework under which the appropriate (economic, health) returns of a GMM release programme can be quantified. Such analyses might be done during or after Phase 3, at a point where sufficiently reliable information about the utility of the GMM is available to allow projections of cost and benefit.

RA for implementation

During RA for implementation, it will be important to review the cumulative RA from earlier phases – were hazards identified fully, were risks characterized accurately and were relevant management measures effective?

The release of transgenic mosquitoes is expected to have effects on target organisms through either the suppression or replacement of local mosquito populations. Failure of intended effects may pose a risk, particularly to human health if the GMM vector control system fails after a release programme is well advanced. By the time a GMM approach is contemplated for implementation, substantial efficacy and biosafety performance data will be available. However, a remaining uncertainty may be related to long-term performance. The potential for evolution and adaptive processes could, for example, encompass the evolution within the target mosquito population of resistance to the transgene function, the evolution of the disease pathogen to resist transgene function or changes in host range of the target mosquito species. RA for Phase 4 must take into account whether any specific surveillance plans need to be put in place for ongoing monitoring of GMM effects. In this regard, plans for ongoing monitoring of GMM efficacy in Phase 4, which is relevant to safety for human health, have been discussed in Section 2. Efficacy evaluation.

RA should include predicting the likely manifestation of any potential resistance (Alphey, Bonsall & Alphey, 2011). This will be highly dependent upon the particular GMM technology under assessment. For example, while a small number of selectively advantageous genes released into an environment might not be expected to persist due to chance (Fisher, 1922; Kimura, 1962), RA for self-limiting approaches should consider whether the mass release under Phase 4 might introduce a selection pressure into an environment that could drive the evolution of novel biochemical or behavioural resistance to the GMM effect. Mutations that confer resistance to insecticides are well known, and it has been demonstrated that mutations favouring resistance can be present in populations before the start of a control intervention programme (ffrench-Constant, 2007). It should be noted that, in many locations, the risk posed by the development of resistance to GMMs might be evaluated in the context of the known risk of insecticide resistance.

Although the possible secondary effects of GMMs may theoretically be extremely broad, RA and RM need to be science-based, proportionate and directed at specific hazards. In particular, the effects on the phenotypic, behavioural and population-level characteristics of the modified mosquito (tables 3.1 and 3.2) on the target population should be reassessed within the scope of risks associated with full public health implementations. The RA for Phase 4 also should identify GMM characteristics that might change as a result of mass production and impair the effect of the GMMs, including selection for altered development rates, size and marker expression. Consideration should be given to the quality control standards for GMM characteristics and procedures (for example, in rearing mosquitoes for release programmes, determining sex ratios for release, etc.) to ensure that processes remain relevant to the RA assumptions throughout the release programme.

Extending the assessment of the effects of the transgenic mosquito on NTO should be considered in preparation for implementation. GMM releases could lead to altered ecosystem functions through trophic effects, such as the role of mosquito larvae as food for predators. Under releases of GMMs aimed at population suppression, alterations (reduction) in target population sizes are expected and, hence, potential alterations in species interaction strengths would be anticipated. In contrast, population sizes might not necessarily be altered under population replacement strategies although the transgenic modification might affect mosquito behaviour or phenotype.

As noted under Phase 3, the likelihood and potential impact of unintended transboundary movement should be assessed. In cases where there is reasonable potential for transboundary movement through either natural or human-assisted mechanisms, it would be appropriate to seek the views of authorities in neighbouring countries on hazards to include in the RA.

Several potential risks with regard to human health should be considered in RA for Phase 4. The release of transgenic mosquitoes may lead to a concern that existing control measures may be reduced, either as people become more lax about personal and household mosquito control efforts or as governments look for cost savings. The implications of a potential reduction in conventional vector control to mosquito population dynamics, human health and to the wider receiving environment require appropriate RA and RM.

The possibility of resurgence of disease when immunologically naïve human populations are exposed to disease after a prolonged period of low incidence is a concern that should be assessed in post-implementation monitoring. This is not unique to GMMs. For example, concerns were initially raised about the possibility that insecticide-treated bed nets (ITNs) might increase mortality in older children through delayed acquisition of immunity to malaria. Empirical evidence from a community-randomized controlled ITN trial in malaria holoendemic western Kenya found no evidence of compromises in human immunity to blood-stage antigens in young children after two years of ITN use (Kariuki et al., 2003) and no evidence of increased all-cause mortality in older children six years after ITNs were provided to children (Lindblade et al., 2004). However, observations of increased susceptibility in older children and adults following long-term use of ITNs have once again raised this question (Trape et al., 2011).

Risk management

RA will determine the need for RM, and, as mentioned above, it may be determined that RM will require tracking of metrics that would trigger a mitigation plan. Post-implementation surveillance may be considered to address remaining uncertainties identified in the RA or to confirm that the conclusions of the earlier RA were accurate once large-scale and long-term open release had taken place. Thus, monitoring and surveillance activities may comprise a key component of the RM plan in Phase 4. By this phase, necessary monitoring methods will need to be easily scaled up and applicable in the field.

Post-implementation monitoring should focus on the appropriate effects and variables (based on data from prior RAs), the duration of the surveillance, the geographical limits to surveillance and the methods by which to measure the effects. Plans should incorporate appropriately designed surveillance procedures to allow effective risk mitigation decisions to be taken when needed, but must take into consideration whether and when it will become impractical to maintain active surveillance as the GMM become ubiquitous under self-sustaining approaches. The RA should establish and delimit appropriate time intervals when the impact and continued safety of the GMM technologies should be reviewed. The post-implementation surveillance method and risk mitigation measures should also be reviewed at appropriate intervals as population levels change.

Mitigation strategies will depend on specific conditions, but might include options such as halting releases in the case of self-limiting GMMs, maintaining public access to conventional disease and

vector- control methods, or designing stopping or recall mechanisms into the technology, such as greater insecticide susceptibility than present in local mosquitoes. The appropriate regulatory structures, mechanisms and methods need to be in place as an integral part of the RA to ensure that clear lines of responsibility are delineated on post-implementation surveillance and risk mitigation, should these be required.

If indicated by RA, implementation programmes should plan for the potential of adaptive processes in the GMM or target population, and management plans should describe the conditions under which mitigation will be undertaken. Quality control in rearing facilities should continually check for any signs of the failure of mechanisms integral to the efficacy of the GMMs or factors that could make control more difficult. RM should include any additional case-specific surveillance methods to monitor transgene activity within the GMMs that were identified by RA as necessary to the decision process for risk mitigation.

RM plans should draw on the results of the RA to determine the need for and design of monitoring to observe the key environmental impacts identified by the CBD (2012):

- effects on biological diversity
- vertical gene transfer
- horizontal gene transfer
- persistence of the transgene in the ecosystem
- evolutionary responses (especially in target mosquito vectors or pathogens)
- unintentional transboundary movement.

However, there should be a rationale in each of these cases whereby monitoring focuses on valid concerns arising from the RA. A plan for case-specific post-implementation surveillance of GMMs should take into consideration any key species for which there is evidence of harmful interactions in order to assess the impact, risks and benefits once a GMM-based control programme is underway. Key species may include those in the main food web interactions and any endangered species listed in national regulations. General surveillance approaches are unlikely to be effective or informative in determining the need for risk mitigation.

In the case of GMMs, the public health implications impose an additional obligation to ensure that the transgenic technology remains efficacious and poses no additional risks, so health monitoring of human populations in the release area should be carried out to ensure the expected levels of efficacy have been achieved. It is anticipated that an appropriate disease surveillance programme could be provided in the context of ongoing national disease control programmes (Section 2. Efficacy evaluation). RM may require that certain conventional disease control practices continue and it may be necessary to integrate the GMM technology into these conventional strategies.

The release of GMMs provides different, but not entirely novel, issues to those for GM plants. Arguably, the most important biological difference is the possibility for autonomous dispersal.

However, appropriate biosafety assessment (Table 3.1) will provide the fundamental information for appropriate RM options. Precedents dealing with biological control and conservation of biodiversity provide additional relevant insights into how the potential for transboundary movement may be managed (Section 5: Regulatory frameworks). Further, there are analogies with biosafety management associated with the release and use of vaccines based on GM viruses or bacteria, where individuals are inoculated with a vaccine and disperse into the wider receiving environment. Establishing the broader environmental risks of GM vaccine shedding rates is of particular relevance. The equivalent for GMMs would be the assessment of dispersal and replication rates (Table 3.1) in the wider environment following an open release (Table 3.2).

The mass rearing and release of transgenic mosquitoes may have consequences (and associated risks) related to cross-border movements and spread. RA of open release trials and post-release implementation of GMMs must consider surveillance to establish the likelihood and consequences of mosquitoes spreading across international borders. This could have ecological consequences, but since most management activities would be national responsibilities, it would be important to consider how neighbouring national authorities would plan and carry out RM actions, including the appropriate surveillance that might be needed. The movement of transgenic material across national/international borders is governed by well established RA and RM procedures, (under the Cartagena Protocol on Biodiversity). Parties bound by the Cartagena Protocol (and its instruments) are expected to carry out the movement of transgenic material (to both Parties and Non-Parties to the Protocol) in accordance with the objectives of the Protocol (Section 5. Regulatory frameworks) and other regional agreements, such as the RSPM 27 of the North American Plant Organization (NAPPO, 2007).

3.8 Consider the need for independent safety review

The establishment of independent safety review groups or the formulation of GMM biosafety regulations for consideration by existing review groups (local bodies such as Institutional Biosafety Committees,[28] national scientific, environmental and public health advisory bodies, and regional or supranational agencies) is recommended. Such groups can provide oversight of the RA and RM within each phase of testing and provide independent scientific advice on the risks of GMMs to human health and the environment.

3.9 Biosafety capacity

The successful implementation of GMM interventions requires transparent, focused, proportionate and credible biosafety assessments. National safety review groups, capable of providing appropriate independent guidance and overseeing all facets of testing and implementation, will be important for biosafety assessments of GMMs and for decisions on appropriate levels of RM. National-level biosafety boards should draw on available expertise across a wide range of scientific, environmental

[28] NIH Office of Biotechnology Activities Institutional Biosafety Committees: http://oba.od.nih.gov/rdna_ibc/ibc.html, accessed 25 May 2014.

and economic disciplines, for example, as provided in the CTNBio in Brazil[29] or CIBIOGEM[30] in Mexico (Ramsey et al., 2014), to assess the risks of GMM technologies. Stakeholder groups affected by releases provide the key to community values and concerns relevant to potential releases of GMM and they should have a consistent and strong voice within both biosafety and benefit analyses associated with the testing and implementation of GMMs.

The decision-making bodies approving biosafety testing should have the capacity to formulate the risk problem, to define appropriate endpoints for risk, to interpret the character of the component sources of risks, to interpret the quantification of risk components, and to understand the efficacy and uncertainty related to proposed RM measures. Where this capacity is not available at a national level, efforts should be made to obtain independent international expertise, and to strengthen the necessary national expertise in the longer term.

3.10 Conclusions

The assessment of the safety of GMMs for human health and the environment should follow a phased approach moving from laboratory and cage experiments through to open-field releases. RA and RM at each stage should provide sufficient information to determine whether a decision can be justified to allow trials to move on to the next stage. This ensures a workable and defined protocol to follow in the development of appropriate decisions for each further testing stage. National regulations governing biosafety, RA and RM must always be followed. Broader international guidelines may suggest some additional aspects of risk analysis that could also be useful, and international obligations on biosafety may also apply in many countries (Section 5. Regulatory frameworks). The decision to move forward with further testing will involve the appropriate oversight and regulatory bodies at each phase.

Not all the considerations described above will be universally relevant to all types of GMMs. It is important to emphasize that RAs should proceed on a case-by-case basis and be proportionate to the particular phase of testing. Defining the potential extent of harm that could be caused to the environment or human health by GMMs, identifying the risk level (hazard by exposure) and developing risk mitigation plans provide the framework in which to undertake the RA. RA of novel GMM technologies should be set against the risk of a relevant alternative comparator. The range of comparators for GMMs at the various testing phases should reflect the range of dimensions of mosquitoes individually, and in populations and control systems, which may give rise to risk concerns at each phase. Comprehensive evaluation of GMM implementation, following trials focusing on safety, should be considered in a broader benefit-risk analysis, and the RA and RM plans form only one component of this broader analysis. Ultimately, decisions must be made on the acceptability of the overall risk, taking account of available and practical RM actions.

[29] Brasil. Presidência de la República Lei No. 11.105, de 24 de Março de 2005: http://www.planalto.gov.br/ccivil_03/_Ato2004-2006/2005/Lei/L11105.htm, accessed 25 May 2014.

[30] Comisión Intersecretarial de Bioseguridad de los Organismos Genéticamente Modificados (CIBIOGEM): http://www.conacyt.gob.mx/cibiogem/index.php/cibiogem, accessed 25 May 2014.

Table 3.1 Example parameters that may be relevant in laboratory studies (phases 1 and 2) as part of the RA for transgenic mosquitoes[a]

Parameters	Example hazards	Assessment methods	Assessment endpoints
Female fecundity **Oviposition rate**	Increased vector abundance	Cohort experiment; life table analysis	Is it limited by population density and/or individual physiology? Is there a significance difference?
Egg development rate **Larval development rate** **Pupal development rate**	Increased growth potential; reduced predation	Cohort experiment; life table analysis	Is there a significance difference?
Egg survival **Larval survival** **Pupal survival**	Increased vector abundance	Cohort experiment; life table analysis; population level modelling	Is it density-dependent? What is the type of density-dependence? Is it under/over-compensatory? Does it differ significantly?
Adult emergence	Increased vector abundance	Cohort experiment; life table analysis	Does the timing of adult emergence differ significantly?
Adult size	Increased vector fitness	Cohort experiment; life table analysis;	Is adult size significantly different?
Adult survival	Increased vector activity; more effective mating potential; increased biting efficiency for females	Cohort experiment; life table analysis; population level modelling	Is it density-dependent? Is it significantly enhanced/diminished by the modification?
Mating strategy	Increased vector abundance; separation of GM and wild types	Cohort experiment	Is there assortative mating? Are there costs to male/female gametes? Does the modification affecting mating competitiveness?
Sex ratio	Increased female abundance; increased biting potential if more females	Cohort experiment; life table analysis	Is the sex ratio substantial different from the null expectation?
Flight ability	Increased vector activity; more effective mating potential; increased biting efficiency for females	Cohort experiment; physiological experiment	Is flight duration or distance significantly different?
Biting rate	Increased disease transmission	Cohort experiment; physiological experiment	Does the feeding rate differ significantly?
Vector capacity	Increased disease transmission	Cohort experiment; physiological experiment;	Is the capacity to harbor pathogens significantly enhanced/diminished?
Insecticide resistance	Increased vector abundance	Standard insecticide dose response testing procedures	Is it expected to alter the competitive status of transgenic lines significantly? Does it make transgenic lines significantly less amenable to conventional control?

[a] The RA should focus on the hazards (changes that may lead to harm as a result of the genetic modification), the (experimental) methods to measure this and the exposure assessment. References to 'differences' mean differences between the transgenic strain being tested and the appropriate comparator.

Table 3.2 Example parameters that may be relevant in open-field studies as part of the RA of transgenic mosquitoes[a]

Parameters	Example hazards	Assessment methods	Assessment endpoints
Population size	Increased vector abundance; ecosystem disruption	Field population monitoring; population level modelling	What is the impact of the release? Relationship between release rate, timing, method and outcome?
Density dependence	Increased vector abundance; ecosystem disruption	Comparator studies at range of densities in laboratory; field population monitoring; population-level modelling	Does the transgenic strain differ significantly in the role of this ecological process?
Spatial distribution	Increased vector abundance; ecosystem disruption	Field population monitoring; population-level modelling; life-table experiments	Limits to the spread of the transgenic organism? Rate of spread of the transgenic insect, under a range of conditions?
Vector capacity	Increased transmission per bite; increased biting rate	Comparator studies; post-release monitoring	Is the capacity to harbour and transmit pathogens increased?
Behavioural resistance	Change in behaviour that avoids, or reduces efficacy of, conventional management	Comparator studies; cohort studies on behavioural changes in different life stages; post-release surveillance; population-level modelling	Under field conditions, what limits the appearance and spread of resistance due to mosquito behaviours? Is there potential for assortative mating in the field?
Biochemical resistance	Change in physiology that avoids, or reduces efficacy of, conventional management	Comparator studies; cohort studies on physiological changes in different life stages; post-release surveillance; population-level modelling	Is the likelihood or rate of resistance development enhanced in transgenic mosquito strains?
Mass rearing quality indices	Quality of released insects is different from planned, affecting negative outcomes	Cohort experiments; comparator studies before release; operational design and audit; pre-release monitoring; post-release monitoring	Do specific aspects of released mosquito quality affect mosquito densities, pathogen transmission and transgene stability?

[a] RA should build on evidence regarding potential hazards obtained during Phase 1 and Phase 2 trials, the methods to measure these hazards and exposure assessments. Comparator studies aim to compare the GM mosquito with a conventional (non-modified) counterpart.

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4. Ethics and public engagement

Summary: Respect for communities should be an overarching ethical goal in GMM trials. Individuals who satisfy the criteria of "human subjects" must be protected according to internationally recognized standards (Section 5. Regulatory frameworks). GMM research also should recognize ethical responsibilities that extend beyond these standard compliance criteria. Public dialogue and outreach are important for realizing research goals, especially in the development of new technologies. Sincere and well-developed engagement can help to direct technical goals, reduce the chance of a misunderstanding of the science needed to meet the goals, and improve the performance of the research project in both technical and social contexts.

Researchers will interact in the course of field testing with different public groups, ranging from those living within the trial site and directly affected by the conduct of the project to third parties interested in the research activities. GMM projects will have ethical responsibilities to people living within a trial site, even when these people are not, in a traditional sense, subjects of the research at hand. Researchers should initiate ethics and engagement efforts during phases 1 and 2, in order to ensure that the goals and methods of the project are well defined and communicated and meet genuine stakeholder needs. Internationally accepted standards for the participation of human subjects in research may apply under certain conditions in small trials with entomological endpoints in Phase 2, but will become more prominent in Phase 3 trials with epidemiological endpoints. Beginning in Phase 2 and expanding in Phase 3, community engagement activities are intended to address ethical responsibilities beyond the formal permissions required at the individual level (informed consent) and the governmental level (regulatory compliance). The concept of "community authorization" entails providing those living in the trial site with methods to give or withhold agreement for trial activities, and to identify elements that they believe to be important for the research to continue. During field testing, scientists also should expect to interact with third parties who express interest in the activity and its outcomes, both to ensure that the project is well understood and to avail the project team of information and insights that such interested parties might provide. However, given the diverse range and varied degrees of interest of third parties, there is not the same level of obligation to seek them out proactively to ensure that they are informed about the project, as is the case with those directly affected. In Phase 4, the responsibilities for implementing GMM technologies and interacting with affected individuals will most likely shift to the relevant local, regional or national public health authorities.

There are aspects of ethics and engagement that may require special skills and training which biologists, medical personnel or public health specialists would not normally be expected to have. Engagement with people living within the field sites may require specialized knowledge of local culture and institutions. In addition, engagement with third parties may require broader communications and negotiation skills. Adequate time and resources must be allocated within the project plan to support these activities.

The success of scientific and public health endeavours can depend upon good will, cooperation and support from diverse sectors of the observing public. Compliance with regulatory requirements that govern the conduct of research, including those concerning human subjects (Section 5. Regulatory frameworks), is mandatory. However, there is ample evidence that simply conforming to regulations and institutional policies does not always satisfy public expectations and researchers' obligations. Beyond the context of regulatory review, the word "ethics" calls attention to concepts of right and wrong, and can imply a standard higher and more rigorous than that of civil authority. Regulations,

laws and organizational policies dictate standards and procedures with which individuals and organizations must either comply or face sanctions that can range from warnings or admonishments to the withdrawal of funding, fines, withdrawal of permission to operate or even prison. In contrast to regulatory emphasis on compulsion and compliance, ethics can be understood as *activity* or *inquiry* whose purpose is to shed light on the correctness or justifiability of some conduct. In the context of GMM trials, ethics aims to understand the interests of stakeholders and their various entitlements, rights, other types of claims and obligations, including what actions or activities are required by the principle of respect for communities hosting the trials. Relevant ethical questions include: How should these rights and interests be recognized in a decision for trials to proceed? How can researchers strike an ethically robust balance between the interests and rights of individuals, the collective interests of the host communities and the properly mandated activities of their public institutions? What is the appropriate role for communication and engagement with media, civil society organizations (CSOs) and others that take an interest in the research?

It is not always easy to maintain a clear distinction between the activities of ethical reflection and engagement and those related to regulatory compliance, which have come to dominate the ethics of research with human subjects (Hagerty, 2004; Rollin, 2008). The major global agencies that fund GMM trials require compliance with international standards for research conduct, including submission of protocols for the use of human subjects, as well as biosafety and the use of animals, to appropriate regulatory oversight committees, usually as a requirement of their own domestic laws and regulations (Section 5. Regulatory frameworks). This may cause confusion, since it is common practice to refer to these obligatory requirements as "ethics" requirements and to various regulatory compliance bodies as "ethics" committees or boards. However, researchers should not assume that regulatory compliance also implies that ethical responsibilities have been adequately addressed. Broader ethical issues and responsibilities are expected to arise in the context of GMM trials that are not specifically mandated by administrative law or organizational policies.

4.1 The role of ethics and engagement in science and technology

Scientists have long appreciated the importance of public dialogue and outreach to realize the envisioned results of their research. However, events and developments over the last three decades have led to a renewed interest into ways and means of interacting with scientists and a number of distinct public groups with different attitudes and interests as regards to scientific work. Some of these events have cast science and technology in a heroic light, while others portrayed people in scientific and technical walks of life as lacking in moral sensibility or fellow feeling. Others simply testify to the way that developments in science and technology can grip public attention, occasionally sparking reactions and consequences that the scientists involved never imagined.

The social phenomenon of public reaction to scientific developments has been the subject of numerous historical, philosophical and sociological studies. Ulrich Beck has argued that general public literacy in scientific matters has created a more sophisticated understanding of how advances in the sciences are accompanied by both benefits *and* risks (Beck, 1992). As a result, citizens have become more aware of scientific or technical breakthroughs as potentially controversial. This awareness has been accompanied by the rise of numerous CSOs dedicated to promoting specific causes. The result is a greater willingness for citizens to become involved in promoting those

scientific activities that they see as consistent with their values or opposing technologies that they perceive to be against their values. Public resistance to certain agricultural and food applications of biotechnology, and to some specific applications of nanotechnologies, is seen as exemplary of this new awareness (Mcnaghten, Kearnes & Wynn, 2008). At the same time, scientists themselves have become cognizant of new ways that involving non-scientists in their work can be beneficial. Exceedingly complex problems may require planned activities that engage non-scientists in collaborative or problem-solving roles, rather than considering them solely as subjects. This has led many to envision a new era of science in which many people can become enrolled in cooperative projects as "co-producers" of new knowledge (Haraway, 1989; Wexler, 2004).

Scientists undertaking work on the cutting edge of discovery or technological capability have both "positive" and "negative" motivations for paying attention to the reaction and receptiveness of the broader public. On the positive side, engagement with people not generally considered to be part of the research community can both enrich the research process, and provide access to information and perspectives that would otherwise have been unavailable to people within the research group. It can also be instrumental in achieving the broader impacts that researchers seek. On the negative side, research that comes under public scrutiny can become the target of organized opposition that has the potential to frustrate not only the application of the science, but even the research process itself. It will not be possible to avoid such opposition in every case. Sometimes opponents of science and technology are simply pursuing interests that are genuinely contrary to the advancement of a given technical project. Sincere and well-developed engagement that acknowledges and demonstrates respect for these perspectives may reduce the chance that opposition is based on a misunderstanding of the science or of its technical goals. In a more positive spirit, it can demonstrate respect for the communities involved in testing the new technologies and may sometimes result in changes or modifications to a research project that researchers view as beneficial.

It is especially important for scientists conducting studies likely to attract significant coverage from the media to consider how their work might be beneficially or detrimentally affected by rapid and broad engagement and interaction with members of the public who have no training in their disciplines or methods. Stories may be disseminated either through traditional media such as newspapers, television and radio, or through new outlets on the Internet and social media. Ordinary word of mouth can also effectively spread a widely shared impression of research goals, intended applications and methods, especially within village or urban settings. Such broad representations of science can have the beneficial effect of expanding opportunities to obtain key informants, participants and partners. However, they also can spread misrepresentations, suspicion, distrust and antagonism to a scientific research project.

4.2 A strategy for ethical engagement

Respect for communities should be an overarching ethical goal in GMM trials. Although there is no consensus among research ethicists about what this requires in practice, the activities of community or public engagement may best be understood as opportunities for demonstrating respect for the communities in question. A broad strategy for helping research teams to meet ethical responsibilities, and conduct public and community engagement activities will involve ethical reflection, interaction with the host community and a wide range of other interested parties, and

iterative integration of findings from these activities into the ongoing planning and conduct of research. The strategy presented here should be interpreted as a description of processes and goals, rather than as a prescriptive formula. As noted by others, when "research ethics" becomes an activity of ticking boxes for compliance, or slavish adherence to rules, rather than one of thoughtful consideration, the real goals of ethical respect and responsiveness may well be lost (Hagerty, 2004; Rollin, 2008).

The ethics and engagement component of a research programme can be visualized at three levels (Figure 4.1).

- *At the project level, there are reflective tasks concerning the broader social and ethical issues raised by GMM trials that shape specific management goals and elucidate important learning and evaluation opportunities for the research*. Such tasks are by no means unique to research on GMMs; an explicit recognition and articulation of the ethical purposes of a scientific project is especially useful when the research is likely to attract public interest and scrutiny, as is often the case with a new technology.

  Scientists involved in projects moving to field trials should plan to devote time and resources to critical deliberative team activities dedicated to reaching and describing a common understanding of the ethical purpose and rationale of the research as an iterative component of the project plan. Over the course of the research, this task may include interactions with advisory committees and consultants, as well as other scientists whose opinions, views and reflections are sought on an ad hoc basis. As the project identifies candidate field trial sites, these reflective activities should be expanded to include critical deliberations with representatives from the host communities where the research may take place, and may include people from other interested groups in an advisory or consulting capacity. The results of these considerations will form a basis for project communications materials, which should be tailored to respond to the interests and concerns of key stakeholder groups.

  Developing a set of criteria for identifying and discriminating between those who are affected by the research activities through specific interventions or interactions, other members of host communities who have a stake in the trial, and those who may have legitimate but more distant interests at stake, and determining how to respond to ethical obligations in each case, will be a component of the broader ethical reflection needed by the project.

- *The researchers need to anticipate a set of tasks that arise from interactions and effects at the site(s) where field studies are conducted*. Conducting research in host communities brings scientists into direct contact with a number of people, including, but not limited to, those who are research subjects or whose cooperation is necessary for successful completion of research tasks. Within the biomedical research model, individuals who are the subjects of specific interventions or interactions, or from whom identifiable information, specimens or materials are collected are classified as "human research subjects" (see discussion below and in Section 5. Regulatory frameworks). However, within GMM trials, it is likely that there will be additional individuals who do not fall within the typical definition of human subjects but who might still be affected by the conduct of the research. This may include those living near a research project whose daily pursuits and/or livelihood could be influenced by research

activities. Thus, tasks at the community level overlap with, but are distinct from, regulatory requirements for securing appropriate informed consent and other relevant protections, and may also include involving and empowering local populations in key elements of research planning and implementation as well as addressing both real and perceived issues that may arise in connection with the project, including broader socioeconomic impact. These tasks may be thought of, collectively, as "community engagement".

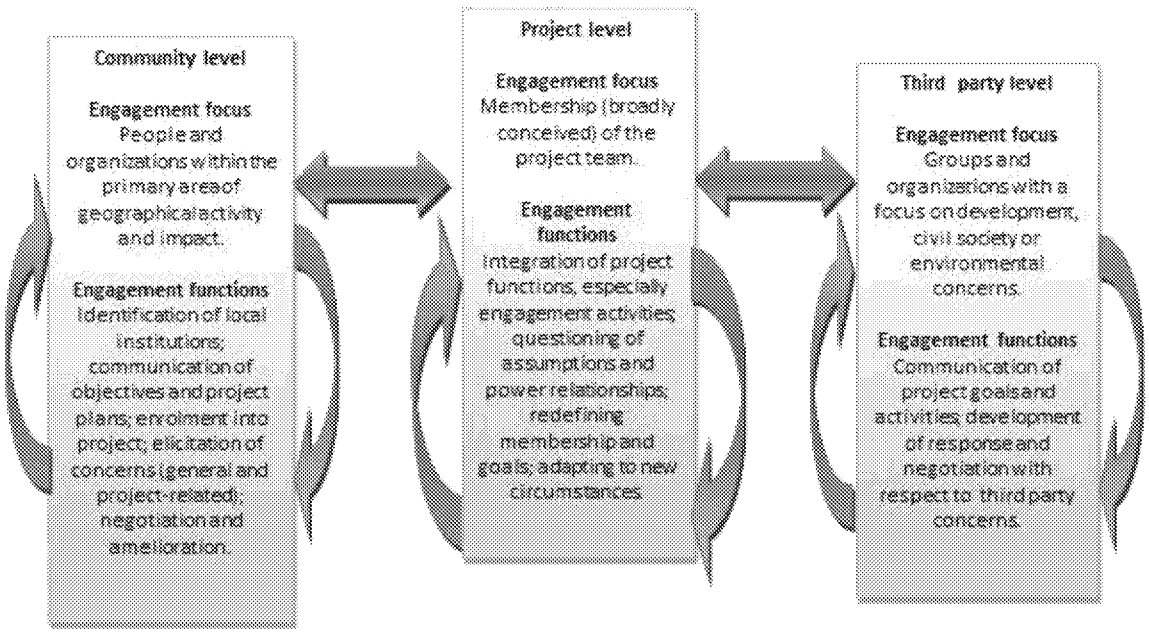
The distinction between people who are affected directly by research and others who are more indirectly interested in its conduct may be operationalized in the way that the relevant ethical obligations are understood. For example, when research involves risks associated with organisms or substances released into the environment, as opposed to contained within experimental facilities, geographical proximity to the site of research becomes an important ethical indicator. In the case of some GMM trials, defining the limits of potential effects may be complicated by the geographical mobility of both people and mosquitoes over time. Such considerations should have been taken into account in a RA (Section 3. Biosafety), which will be helpful in guiding identification of community stakeholders.

- *There will be tasks related to the involvement of individuals and groups who are not immediately affected by the research, including CSOs, the press and the general public.* People living at a distance from the trial may have friends and relatives or even economic interests that they fear could be affected by the conduct of a research project and, thus, may also perceive themselves to be affected by it. Moreover, a much larger community of people may take an interest in the conduct or outcome of research, even if they are unlikely to be physically affected by the trial activities themselves. For example, people who are afflicted with a particular disease (along with their friends and family) have an obvious interest in the outcome of research or clinical trials, even if they are not involved with specific trials. Such groups are likely to be strongly supportive of research intended to improve their condition. In a similar vein, people who care about causes such as protecting vulnerable groups or endangered species may take an interest in a wide range of research activities, and may not be unilaterally supportive of research goals or procedures. Although the nature of responsibilities to such individuals or groups is quite different from those to communities hosting the trial, it is clear that an effective plan for engaging a wide spectrum of interested parties can be critical to the success of research, especially for projects that can be expected to attract a significant amount of attention in the press or monitoring from CSOs.

The plan for addressing engagement should include activities appropriate for each level. Each of these activities should be understood as iterative and sustained during the entire research period, as illustrated by the feedback arrow loops in Figure 4.1. Each group of tasks should be understood as an ongoing component of the research activity, and the research plan should include a programmatic discussion of how tasks in each of these three areas will be carried out by members of the research team on an ongoing basis throughout all phases of the research activity. Researchers must also take into account that communities and third parties may become engaged with each other independent of the project.

One helpful way to use the three levels of activities for planning purposes is to focus on *who* will need to be involved in completing them. Activities at all three levels of engagement involve members of the research team, and will almost certainly involve staff from the sponsoring organizations as well. Meeting ethical responsibilities to the full range of stakeholders in the host community requires a great deal of work "on the ground" in the local areas encompassing the research field sites. As will be explained further below, this may not imply contact with literally every individual in the contiguous area, but it must be understood to require appropriate attention to local forms and mechanisms of representation for those who will be affected by the research activities. This may involve negotiation of the environmental and developmental goals, standards, and metrics for the research. For example, directly affected parties and international civil society groups alike may have a desire to participate in discussions about how risks to biodiversity are measured, or how economic benefits are understood in relation to improvements in public health. One cannot assume that all parties will see any and all forms of economic growth or resource development as beneficial, and investigators should not assume that local communities would always be forthcoming or comfortable with expressing these interests. There may be some areas of overlap between the ethics issues that arise on the ground in interacting with local stakeholders, and the ethics of environment and development that represent concerns of third parties. Some third parties might decide to represent the interests of local people, though the local communities may, or may not, view such representation as legitimate. Anticipating and preparing responses for the issues that are likely to arise in such interactions is an example of something that falls into the category of "broader ethical concerns" to be addressed at the project level.

Figure 4.1 Levels of engagement focus and function



Activities at all three levels will include the following.

- ***Ongoing literature and methodology development.*** *W*hether it be best practices for clinical and epidemiological research, or engaging with communities, nongovernmental organizations (NGOs) or the press, there now is a body of relevant literature that should be taken into account in planning and implementation of a project of the scale required for GMM trials. Appropriate review and application of this information will require, at the project level, participation of team members or consultants with the necessary background and expertise.

- ***Task planning and implementation.*** Based on this literature, those responsible for the ethics and engagement activities will undertake the planning and implementation of project procedures. This may involve staff training, consultations, development of information about the project (including language and culturally appropriate information for use in interacting with residents at field sites), surveys, educational activities, workshops, negotiations, etc.

- ***Documentation and reporting.*** Record-keeping requirements are specified with respect to research involving human subjects. However, it must be stressed that other ethics and engagement activities conducted under the project also should be documented to allow for later reporting, and mechanisms should be developed to accomplish this. Records of ethical deliberations as well as stakeholder interactions and agreements could prove important in the case that challenges to the project arise. Reporting in the form of peer-reviewed articles on

the ethics and engagement activities will enrich the literature and help with the planning of future GMM research.

- ***Evaluation.*** Both internal and external evaluations of how well tasks at each of the three levels are being performed should be part of the plan. One or more members of the project team could potentially do internal evaluations, but the plan should specify such responsibility explicitly. External evaluators can be drawn from project management specialists, as well as specialists in the ethical dimensions of public health.

- ***Iteration.*** Evaluation should lead back to methods development and planning. This process will be repeated periodically as needed.

4.3 Activities at the project level

Most scientists view their work as having value and a social purpose, and this may be especially so for those conducting research on public health and disease control. However, scientists do not always articulate the purpose of their research explicitly, or discuss its value with others. Reflection is an activity of both articulating value and purpose, and initiating critical discussion of the project among members of the project team. Reflective activities should encourage openness among the research team to the possibility that the social, medical or public health rationale for a project may not be sufficiently well grounded to warrant its continuation. But more typically, these activities can stimulate constant reconsideration of project aims and research design and methods to ensure a continuous opportunity to bring project activities more fully in line with public health objectives and social goals.

Making explicit the value and social purpose of a scientific research project initiates a broader reflection that serves several key functions. First, an explicit discussion of how research will give beneficial outcomes can yield unexpected improvements in project design. Conducting such discussions with project team members, advisers and consultants increases the range of knowledge and interests that can be incorporated into the research design, and will help to ensure that important strategies or alternatives are not overlooked. This helps researchers avoid losing time by pursuing strategies that may be technically feasible, but cannot be implemented due to their incompatibility with social mores, legal mandates or other elements in the technical infrastructure. Second, public presentations of a project's motivation, goals, and ethical vision and explicit articulations of the ethical considerations that guide the scientific work, and its relationship to various social goals, disseminates this thinking to a broader audience and may prove helpful in winning the trust and cooperation of host communities. Finally, the public record that is created by documenting how and why the science was done creates an opportunity for others to learn. Canada has pioneered approaches to embed such activities within large-scale research projects dedicated to biological research (Castle & Culver, 2006; Coward, 2006), and some of these may serve as useful templates for GMM trials.

It is especially appropriate for researchers working on GMMs for disease control to engage in and support such reflective activities within their trials. There is a well-established record of conflicting views on the most appropriate strategy for addressing persistent global health problems such as malaria. Some authors express extreme skepticism about initiatives that propose "big science"

approaches (Packard, 2007), as opposed to improved implementation of simpler and more accessible local solutions. Parties involved in GMM research should be aware of this history and be willing to reflect critically on the role of their own project in this enduring debate. Additionally, the use of GM approaches on animal species provides these projects with a second linkage to research traditions involved in well-established debates (Thompson, 2007).

Therefore, it is recommended that projects on GMM research include structured ethical reflection as a specific and planned activity, and that both time and resources be allocated to ensure that this is not neglected. It may be fully appropriate to schedule these activities in conjunction with key project milestones. These should incorporate some form of public reporting on thinking within the project, including "lessons learned". Such public reporting might take the form of peer-reviewed publications in appropriate ethics or policy outlets, seminars or workshops, updates on the project website, etc. (for example, see El-Sayed et al., 2009; Osrin et al., 2009; Lavery et al., 2010a; McNaughton, 2012).

4.4 Activities at the host community level

To demonstrate the efficacy of GMMs for vector-borne disease control the necessary trials are likely to involve complex designs and will progress from purely entomological trials to trials involving the measurement of epidemiological outcomes associated with GMM releases in defined areas (Section 2. Efficacy evaluation). At different phases of testing, different interactions come into play.

People living at the trial site may be in immediate physical contact with the research team, their buildings and vehicles, and with any materials or substances that are released, intentionally or not, into the environment. For GMM research, this includes the perceptions of people who may see, hear or be bitten by any mosquitoes in the field-testing area. There may be some ambiguity in determining who has the potential to be affected in this sense, as there will be movement of both humans and mosquitoes through the locale and complex opportunities for different types of contact. Experience with GM crops illustrates the need also to consider the possibility that some may have concerns about longer range economic, spiritual or cultural effects. Identifying who may be affected by a GMM trial, and in what ways, is itself a key project level ethics activity.

How should risks associated with GMM trials be communicated?

GMM trials represent a challenge for conventional, individual-focused research ethics, because the associated interventions (the release of mosquitoes) are not administered to individuals, but are literally released into communities. The interventions have their impact at a collective level.

Since mosquitoes are capable of unpredictable movement among locations, it will be impossible, in advance, to identify all persons with whom they might come into contact. Indeed, in the general case of vector biology research it has been proposed that biosafety oversight (Section 3. Biosafety and Section 5. Regulatory frameworks) may be a more appropriate model than individual human subjects protection (Aultman et al., 2000). Lessons may be taken from environmental health programmes, which usually characterize risk in epidemiological terms that make it difficult to describe the exact causal mechanisms of exposure or to translate population-based exposure calculations to the individual level. Such environmental risks typically are not amenable to ethical procedures that presume an opportunity to exit or "opt out" of the risk-bearing situation. What is

more, they raise considerations about the way that risks are distributed across economically, politically or ethnically vulnerable populations—issues of environmental justice. There are no ready analogues to environmental justice in standard human subjects research ethics (Lavery et al., 2003). These similarities suggest that GMM trials, which involve exposure to potential environmental hazards, may need to be evaluated from an ethical perspective that incorporates considerations rarely contemplated within standard human subjects deliberations.

As the ethical evaluation of research places increasingly greater emphasis on the way that a research activity is intended to benefit the parties that will be exposed to risks (Emanuel et al., 2004), it thus becomes increasingly important to involve and empower those parties. This requires that the relevant processes include adequate representation from the host community. Mechanisms to accomplish this will vary according to location and societal norms. In some instances, special measures and innovative organizational activities will be necessary, while in others it will be more important to work with well-established social and political procedures or institutions (McNaughton, 2010). Mechanisms for providing information on risk may need to be tailored to local cultural practices and levels of linguistic and mathematical literacy (Shapiro & Meslin, 2001). Greater attention to these processes in research ethics review can help to avoid circumstances in which host communities are simply passive recipients of activities designed and delivered by others (Crocker, 2008).

Informed consent in GMM trials

Informed consent is a process intended to ensure that human subjects who will be observed or involved in a research activity are fully and explicitly advised of all risks, costs or inconveniences they may bear as a result of participating as a research subject, and voluntarily agree to accept or bear those risks and costs. Some commentators have argued that informed consent will be necessary to ensure that GMM trials are conducted ethically. However, the precise circumstances under which informed consent must be obtained, and from whom, require careful consideration.

Informed consent is universally recognized in research ethics regulations and guidelines as a necessary protection for human research participants (Section 5. Regulatory frameworks). Research ethics guidelines and regulations generally rely on four criteria to determine whether an individual is a research participant, and therefore should normally give informed consent as a condition of their participation: (1) if an individual is directly intervened upon by an investigator; (2) if an individual is deliberately intervened upon via manipulation of the individual's environment by an investigator; (3) if an individual interacts with an investigator for the purpose of collecting data; or (4) if an investigator obtains identifiable private information about the individual for the purpose of collecting data (McRae et al., 2011).

Caged-field trials or open releases of GMMs in the context of a research trial would not satisfy the requirements of the first two criteria, since no individual is intervened upon directly or deliberately, even if they live in close proximity to the cages or release sites. The third and fourth criteria focus on the interactions between investigators and individuals who play some special role in generating or facilitating the collection of study data.

In GMM trials there is a wide range of interactions with the host community, but only a select few that are associated with data collection. In early phase trials, this would pertain to individuals who agree to complete surveys or participate in interviews for research purposes associated with the GMM trial. It would also pertain to those homeowners who agree to the placement of mosquito traps for monitoring purposes, or who permit researchers access to their homes for the purpose of collecting mosquitoes. In particular, mosquito collection in homes for research purposes is likely to be linked to global positioning system (GPS) data, which would be required for spatial analyses of the spread and species composition of mosquitoes after releases. When these GPS data are highly precise, they will effectively tie the associated mosquito data to specific households, thus rendering the data identifiable at this level even if they are not personal in nature. Since it is the household that is identified, and not an individual, the consent of the head of the household or her/his designate is more appropriate than a requirement for all members of the household to provide informed consent. And given the extremely low levels of risk associated with these types of data collection activities, institutional review boards might further consider modifications of normal consent procedures, such as verbal consent or full waivers of informed consent, as long as all other necessary permissions and protections have been secured.

As trials progress from primarily entomological endpoint designs to incorporate epidemiological endpoints, such as incidence of new infections with dengue or malaria, they will require the collection of blood and other forms of clinical data. In both cases, the data collected will constitute identifiable personal information and individual informed consent will be required.

Two general conclusions follow from this analysis. First, simply living in the vicinity of a GMM release is not sufficient grounds to require informed consent from any individual for an open release of mosquitoes. Second, the interactions with individuals and households for the purposes of data collection in trials with both entomological and epidemiological endpoints are likely to give rise to individual, or household-level identifiable data and, therefore, in the absence of specific exceptions or waivers, will require informed consent.

What constitutes adequate authorization from participating communities to conduct the trial?

As described above, informed consent will be required in some circumstances, and it also is expected that GMM trials will require formal authorization by relevant government authorities in recognition of the country's sovereignty (Section 5. Regulatory frameworks). However, these two levels of formal permission may still not fully acknowledge the range of interactions, rights and interests within the host community. Measures necessary to fill this gap can be thought of as being guided by the informed consent goal of protecting the interests of those who will be affected by research. But they may need to use alternative mechanisms for communicating the aims and methods of the science, and the potential risks and benefits of the project at a broader level, and for achieving sufficient assurance that the community has agreed that the research and public health interventions should take place.

Community authorization and informed consent share several key elements. Both promote a deliberative model for addressing ethical issues that arise in connection with research. Rather than relying on strict rules or criteria that must be followed, the deliberative approach mandates that ethical issues be considered before the research is actually undertaken and periodically reviewed.

Both are intended as a mechanism for demonstrating respect for persons who will be affected by a research project or a public health intervention. Both imply "voice", an opportunity to express concerns and to receive replies that are addressed specifically to these concerns. A reply might take the form of assurances or clarification of activities and/or risks, yet for the conditions of voice to be met fully, affected parties must accept the assurances offered as a satisfactory response to concerns. Response also might involve modifications to the plan that relieve concerns, such as additions to RA or RM activities.

Community authorization differs from informed consent in at least three key respects. First, the methods of informed consent that have dominated discussion of research ethics in the industrialized world assume that consent is given or withheld by individuals. When possible, the individual in question is the person who bears the risks, but in cases of children or people who are incapacitated, a third party is authorized to give or withhold consent on their behalf. Community authorization is a procedure intended to elicit agreement on behalf of a group, often a political community such as a neighbourhood or township. Thus, procedures for community authorization more typically rely on norms for group decision-making such as voting, consensus or negotiations with leaders and representatives who are recognized as having the authority to speak on behalf of the community as a whole. Since norms for group decision-making vary widely, it is especially critical that procedures for identifying leaders and representatives, or for interacting with community groups, are based on detailed knowledge of the locale, its traditions and its history of cooperation, exploitation and conflict resolution (Christakas, 1992).

Second, even where there are established leaders and decision-makers in the host communities, GMM trials are likely to involve a wide range of interests spread across a number of different groups, not all of which will be governed by the same leaders. As a result, researchers should be wary of assuming uncritically that any one decision-maker can provide definitive representation of a host community. One key implication for authorization is that, unlike individual informed consent, there may not be one specific mechanism or point in time in which authorization is granted. Instead, it is likely to be more of a judgement on the part of researchers that they have exercised the appropriate level of diligence in eliciting and responding to the concerns of the interested parties and groups, and vigilance in maintaining the necessary commitments and relationships once it is determined that there is a general collective will to proceed. In the absence of a specific mechanism, authorization may represent an accumulation of endorsements or assent by key stakeholders. Collectively, these activities, which are sustained over the full duration of the GMM trial, from planning to post-trial negotiations, constitute community engagement, which is described in more detail below.

Third, unlike individual informed consent in most biomedical research trials, community engagement and authorization by the community for GMM trials will probably not be sufficient on their own to allow trials to proceed. There will usually be a need to secure formal government permission to import the GMMs to be used in the trials and to begin the planned trials (Section 5. Regulatory frameworks). Community authorization may play a role in regulatory decision-making.

Community engagement

Community engagement is fundamental to the process of obtaining community authorization. Engagement and involvement with the communities hosting the GMM trials must be guided by detailed knowledge of the local community, its institutions and common practices. Finding out about the kinds of concerns the community might have, about any past negative engagements, or determining what the community wants/expects in terms of engagement or consent will be important (McNaughton et al., 2010). Such information is best obtained through ongoing relationships and/or extended ethnographic work with individuals from different social classes, gender, occupation and social role. Establishment of the necessary relationships, which will be unique to each setting in which GMM trials will be conducted, will be critical to putting in place an appropriate process of ethical review and engagement, especially in the early stages of testing (Hyder et al., 2009; Marsh et al., 2011). In many cases, particularly in more traditional community settings, community leaders may play a central role in introducing the researchers to the community and its social structures (Tindana et al., 2011) and in providing various levels of ethical scrutiny and permission (Diallo et al., 2005).

At the most general level of description, community engagement is a set of procedures and their motivating ethical goals that aim to develop fair and respectful collaborative interactions with communities around the introduction of a new technology or intervention. It is carried out in a way that protects the interests of the community while permitting the introduction and testing of promising new technologies to improve health. Detailed guidance about what constitutes effective community engagement is still under development. However, one of the first frameworks for community engagement in global health research was developed specifically for GMM research (Lavery et al., 2010b), and is a potentially very useful resource for the design of community engagement activities to support authorization from host communities for early stage trials (Box 4.1). This study also addressed the issue of how to define the community for purposes of engagement, citing two principles: 1) the community comprises at least those individuals who share identified risks associated with the proposed research project; and 2) there may not be a pre-existing and established community as envisioned by the researchers, but rather, the relevant community may take form progressively in response to specific aspects of the research, and to engagement activities associated with the project (Lavery et al., 2010b).

Host communities for GMM trials will most likely have multiple "layers" of authority, such as a municipal council, a Chief, village elders, a chamber of commerce, a farmers' association, or a household. Each must effectively give its permission for a trial to proceed. This permission is seldom determined in a single decision, but rather demonstrated and expressed over time in the ongoing willingness to cooperate and participate with the trial in various ways, or not to actively oppose it.

Box 4.1 Points to consider for effective community engagement

(i) Rigorous site-selection procedures.

(ii) Early initiation of community engagement activities.

(iii) Characterize and build knowledge of the community, its diversity and its changing needs.

(iv) Ensure the purpose and goals of the research are clear to the community.

(v) Provide information about the research.

(vi) Establish relationships and commitments to build trust with relevant authorities in the community: formal, informal and traditional.

(vii) Understand community perceptions and attitudes about the proposed research.

(viii) Identify, mobilize and develop relevant community assets and capacity.

(ix) Maximize opportunities for stewardship, ownership, and shared control by the community.

(x) Ensure adequate opportunities and respect for dissenting opinions.

(xi) Secure permission/authorization from the community.

(xii) Review, evaluate and if necessary, modify engagement strategies.

Source: Lavery et al. (2010b).

Within the community engagement framework proposed by Lavery et al. (2010b) (Box 4.1), items ii–x address specific needs for information or activities that will almost certainly need to be supervised by persons with training in appropriate field disciplines in the social sciences. Persons who are naturally fluent in language, local tradition and customs, and who can translate between the community and a research team while effectively communicating risk are rare. Furthermore, these individuals will need to commit a significant amount of time to activities within the local communities, and these activities will require a significant financial commitment from the project. The composition of the research team should reflect the process for engaging with local communities, gathering this information and integrating it into the project's planning and deliberation process. Depending on the competencies of both project staff *and* locally affected parties, it may be appropriate to include representatives of affected groups within the project's governance mechanisms.

4.5 Activities at the third party level

Those with interests in GMM trials will probably not be limited to individuals and households with the closest geographical proximity to the trial sites. Instead, there may be a wide range of individuals and groups with a legitimate interest in the conduct and outcomes of the trials. Relevant third parties may include the following groups:

- persons associated with global or regional public health and international development organizations, including governments;
- scientists and members of scientific organizations with disciplinary or trans-disciplinary links to research activities associated with field-testing activities, including sciences dedicated to public health and infectious disease;

- persons and organizations engaged in competing approaches to the control of infectious diseases;
- members of organizations focused on promoting the interests and protecting the rights of poor and/or historically marginalized people;
- members of organizations dedicated to the preservation of endangered species, genetic diversity and threatened ecosystems;
- members of organizations with a history of monitoring the role of the sciences in debates over the use of biotechnology;
- individuals and organizations with ties to national, regional and cultural groups active in the areas where field testing is occurring;
- international organizations such as those within the United Nations system.

Some of these groups and the individuals involved with them may have either formal or relatively well-established ways to express views on GMM projects intended for controlling disease vectors and to interact with project staff, while others may not. In light of experiences with the global controversy over GMOs, it is wise from both an ethical and a strategic perspective for any community engagement framework to include mechanisms and procedures for systematically engaging with third parties.

There is not the same level of obligation to seek third parties out proactively to ensure they are informed about the project, as is the case with those that may be affected by virtue of proximity to a trial (formal interactions with government authorities required for regulatory approval are covered under Section 5. Regulatory frameworks). However, interaction with third parties is ethically responsible because the parties listed above have legitimate interests in the conduct and outcomes of GMM field testing. In order to fully satisfy the ethical requirement of respect for the relevant communities, the project team must develop and implement planned activities to consider the interests of third parties and engage with them in a respectful manner. The team must also determine when duties to consider the interests of third parties or involve them in project decision-making or oversight are overridden by more compelling concerns or ethical responsibilities. Engagement with third parties could grow to the point that the cost in time and resources hampers other aspects of the project. The ethical responsibility to inform and engage third parties must be balanced against the need to utilize time and other resources in completing the project's overall goals. Undertaking a process of stakeholder analysis early in the project may be helpful in this regard, by facilitating the identification of third parties most likely to influence the success of the project (Bryson, 2004).

In addition to being ethically responsible, engagement with third parties may be of strategic importance to the project's success. Third parties may have information or comments that can materially improve project activities. Their support and good wishes may contribute to a variety of activities ranging from securing funding or regulatory approvals to facilitating interactions with other scientists, suppliers, publication outlets and local officials. Strategically motivated interactions with third parties are an inherent part of science (Latour, 1987; Collins & Pinch, 2002) and should not be regarded with skepticism. Scientists are adept at some strategic interactions, especially those relating to their disciplinary colleagues, but can be inept at others. In the history of agricultural biotechnology, for example, many avoidable misunderstandings and much mistrust occurred. This

was because scientists in both public and private sector positions were insensitive to the fact that consumers and environmental advocates perceived themselves to have legitimate interests that were being neglected in the process of developing transgenic seeds and animal drugs (Charles, 2001). What is needed for strategic management is a broadening of the perspective that scientists bring to their research to include an effort to understand and then interact with people holding perspectives on the research project that may initially seem to be unrelated to, or at odds with, those of the scientific team.

The mechanisms for accomplishing this kind of broader outreach and engagement are still not well understood. One lesson that is now well established is that this kind of activity should *not* be conceptualized solely in terms of public education, or of simply informing third parties of things that the researchers know about GMM and vector control. Communications launched with this so-called "deficit model" of public engagement have been shown to not only fail, but also to substantially increase opposition and mistrust, (Klienman, Eisenberg & Good, 1978; Wynne, 1996; Hansen et al., 2003; Gjerris, 2008; Toumey, 2009). Rather, it is crucial to develop mechanisms of interaction with third parties that are based on what Pielke (2007) calls "the honest broker" approach. The keys to this approach are to first recognize that third party interests reflect values-based standpoints that inform the way that a scientific research project is going to be seen as either responsive to a problem or, alternatively, as contributing to a problem. Second, it is critical to develop communication materials about the project that are framed in response to these values-based perspectives. Putatively "neutral" descriptions of projects may fail to provide information that allows third parties to gain a clear understanding of why the research is relevant to them. If such materials are disseminated to parties that are already suspicious or skeptical of a project, they can actually exacerbate feelings of mistrust. Finally, it is important to present a picture of the research that includes both strengths *and* weaknesses relative to the values perspective that would motivate a third party to take an interest in it. While such a communications strategy should strive to be complete, it should also be sensitive to the need for concise treatment focusing on the problem at hand.

Thus, projects should include a general communications strategy based on Pielke's principles (2007). These communications can be disseminated through an array of media, including the Internet and through presentations at professional or public meetings relevant to key interests (e.g. environment, public health, poverty and development, science policy). Other strategies for engagement with the public utilize universities, television and science museums (Wilsdon & Willis, 2003).

Once a public engagement strategy has been launched, there should be opportunities for follow-up activities. These could include provision for the submission of comments and questions, but might also involve more extended interactions. It is crucial that third parties invited into engagements of this sort are not made to feel that they are being placated, simply tolerated or, even worse, that the engagement is simply a stalling tactic with little genuine opportunity for third parties to have any substantive input (for example, Griffiths & Steinbrecher, 2010).

Just as with discharging responsibilities for engagement with those immediately affected by research, engagement with third parties will be more effective if researchers and/or consultants with specialized skills are part of the project team. As such, there should be a component of the research activity that is designed and dedicated to third party engagement. It should be equipped

with adequate personnel and budget, and this should include some time and energy commitments from leaders in the biological science component of the research. This is an important point for funders of GMM trials to understand, as these types of communications activities are not a standard component of research budgets.

4.6 When should ethics and engagement activities take place?

The timing for tasks such as securing authorization and support from those that will be affected by the research will probably be implicit within the nature and goals of the activity. It is important to stress that these procedures must be organized and conducted before they have an actual impact on affected parties. However, agreement secured too far in advance will simply need to be renewed, as people change their minds. Similarly, there will be a need to plan efforts to revisit these tasks over the course of the project.

Phase 1–2 trials

The traditional model of engagement and outreach for scientific research that held sway for the first half of the 20th century would have envisioned little need for engagement activity at the early stages of research, up to and including field testing for agricultural or public health interventions. According to this view, the public did not need to be particularly aware of a research activity until their help or cooperation was needed in actually undertaking a large-scale intervention. However, as cognizance of risks to human research subjects grew, and standards for procuring cooperation and consent began to develop, researchers recognized that there were key activities needed to inform and involve affected parties, even at this relatively preliminary stage of research. While it is less likely that major controversies would erupt before field testing, the complexity of GMM research suggests that it is advisable for researchers to commence the "broader issues" engagement component as early as possible, and certainly before Phase 1 proof-of-concept work has been completed. This could be done, for example, by collaborating on a publication that discusses the ethical rationale behind proof-of-concept work. Need for stakeholder engagement and community authorization activities would be expected to arise in Phase 2 of the proposed GMM-testing pathway.

Box 4.2 Disruption of the testing of male sterile mosquitoes in India

Public health scientist Robert S. Desowitz described an episode in one of his books written for a popular audience that is instructive to consider: "On a morning in 1975, a van bearing the blue-and-white logo of the World Health Organization on the door—a snake caduceus through a global map—drives into the village center. The villagers, who have a fear and loathing of snakes, regard the serpent van suspiciously. They begin to be even more suspicious when a peculiar collection of men emerges from the van—a few undoubted Indians, some strange Orientals, and some very white white men. An angry murmur of astonishment passes through the gathered group of villages when these men remove large mesh-covered cages from the vehicle, open the cages—and out flies a cloud of mosquitoes. Without a word of explanation, the snake and mosquito men then return to their vehicle and drive away. Several weeks later, the snake van appears again in the village and once more the strange foreigners release a cloud of mosquitoes from the cages. The crowd reacts—chasing the men into the van, which makes a hurried escape. A month or so later the vehicle appears again. The villagers burn it." (Desowitz, 1991:89.)

Desowitz (1991) writes that the villagers complained to parliament, and that parliamentarians accused the American scientists of conducting an experiment in biological warfare. It was later confirmed that these suspicions were entirely unfounded (Powell & Jayaraman, 2003).

A few key episodes in field-testing have demonstrated how poorly executed public relations and engagement strategies can sabotage research efforts, sometimes having extremely long-lasting effects. Particularly relevant to GMM field testing is an episode that occurred in conjunction with a field release of male sterile mosquitoes as a component of research on vector control in India (Box 4.3). A cooperative project involving scientists from India and the USA, among others, was conducting field trials with male sterile mosquitoes as basic research that could be adapted to a number of disease control situations. However, suspicions were raised both locally and in the national press about the nature and intent of this research, which were exacerbated by poor communications, and the project was unable to continue (Anonymous, 1975). This episode has been repeatedly cited by those who warn that GMM field testing must be accompanied by effective efforts to engage both local individuals in areas where field trials will be conducted and also activists self-identified as promoting pro-poor, pro-environmental issues and democratization of science initiatives (Benedict & Robinson, 2003; Curtis, 2006; Knols et al., 2007).

These incidents illustrate why adequate plans for communication and engagement are important even at the early field-testing stage. This brief history of unfortunate episodes testifies to the potential for misunderstandings that can cause irreparable damage to specific research efforts. What is more, knowledge of these cases inclines some public advocates to be highly skeptical of the intentions and ability of scientific research efforts to respect and involve an appropriate cross section of stakeholders, affected parties and representative members of the interested public through key phases of planning and executing field trial activities. While engagement activities complement and support other project activities that are dedicated to the anticipation and management of risks or regulatory compliance, the history of field trials gone wrong shows that these components have a purpose that is independent of RM and regulatory compliance. Protecting the integrity of the trial, and the ability to work both locally and in a global culture of support for the project depends on an effort of good faith to engage social and ethical issues.

It is recommended that investigators work cooperatively with their institutional committees, including committees responsible for research ethics review, and with the host communities to

avoid miscommunications and misunderstandings that could undermine trust and transparency. When field releases begin, communications should be careful to explain that trials are research activities intended to test the efficacy of a new technology, a protective effect is not assured, and the community must continue to employ other available methods to protect themselves from disease transmission.

Additionally, as described above, certain individuals may meet the criteria of research subjects, even in the case of small entomologically focused Phase 2 studies, as a result of interventions or interactions, such as the collection of specimens, data and private information. Unless determined otherwise by the relevant institutional ethics committees, it may be presumed that informed consent should be obtained from such individuals in advance of the collection of data.

Phase 3

Efforts to engage potentially affected parties will expand in anticipation of larger Phase 3 trials. In addition, human subjects issues will become more prominent, especially in trials seeking to evaluate epidemiological efficacy where measurements of the incidence of infection and other medical information will be required. Such trials are likely to assign groups of individuals to treatment and control clusters, rather than to involve a randomized distribution of individual subjects. Some individuals in clusters may have no direct contact with researchers, and their personal identities may not be relevant to the research process. For these individuals, the above argument that they are not subject to a direct effect of the research can be made. However, in Phase 3 trials for epidemiological endpoints, data collection designed to shed light on the health impact of GMM releases will require the selection of individuals within the community for the purpose of securing the necessary data or personal information, for example, through surveys or blood samples, even in large-scale trials. In these large trials, procedures would resemble those of vaccine trials, which typically require multiple interactions with individual participants over the course of the trial, and which also provide appropriate contexts and moments for securing and reaffirming informed consent. An important difference between GMM trials and vaccine trials is that in GMM trials, participants would be consenting (or not) to the collection of data, *not* to the intervention itself (the GMM release), which would not affect them at an individual level.

Planning for scaling up community engagement activities should commence well in advance of Phase 3 trials. Community engagement at this broad scale will be challenging because of the inherent difficulty in replicating across extensive and diverse populations the high-quality, trusting relationships between researchers and stakeholders that were possible through ongoing personal interactions in Phase 2 trials. For large and multi-site trials, additional mechanisms of public engagement, perhaps including social and mass media, may need to be invoked to reach and obtain feedback from a broader community than would have participated in Phase 2 testing. Such mechanisms also may facilitate monitoring of public opinion and demonstrating trial acceptance. Additional representational methods may need to be considered for obtaining community authorization, and it will be important to ensure the validity of these methods. It has sometimes been the case in cluster randomized trials of the type envisioned for Phase 3 GMM trials, that the consent of the relevant cluster population(s) has been sought from a "guardian," such as a village elder or elected official, and perhaps without the knowledge of those involved in the trial, in order to avoid the possibility of changing behaviour or otherwise biasing the control clusters (Edwards et

al., 1999). A meta-study of such trials suggests that ethical issues have not been sufficiently clarified, and that ambiguities leave open the potential for ethical abuse with respect to the level of understanding and agreement that is required from the study population (Weijer et al., 2011). Thus, even in the context of large-scale trials, appropriate community engagement and community authorization procedures will be expected to adhere to the principle of respect for communities, aiming for widespread understanding and ongoing endorsement by those living at the trial site.

Another question that will be encountered in Phase 3 trials concerns the type of care that should be provided to control groups during a randomized controlled trial. The ethical debate generally focuses on whether the control group should receive a "proven effective" treatment, the "locally available" treatment, or some other treatment (van der Graaf & van Delden, 2009). It is not clear that "standard of care" is even an appropriate concept for GMM trials, since the concept has been imported uncritically from drug and vaccine trials that are different in several ethically relevant ways. However, it is likely that research ethics committees will require investigators to design trials to ensure that other forms of vector control, or other treatments that reduce the amount of human infection, and could therefore influence the background level of pathogen transmission, will be provided. This type of requirement could have a significant impact on the trial's design, since low transmission levels will make the efficacy of GMMs more difficult to measure. For example, in GMM trials for malaria control, one ethical question might be how actively to promote the concurrent use of bed nets. Another such question will arise if and when a malaria or dengue vaccine becomes available for public health intervention.

Further work will need to be done to determine the most appropriate way to conceptualize these "standard of care" issues for GMM trials. But, as these specific aspects of ethical trial design are being developed, investigators should prepare appropriate strategies for addressing them, along with the rationales for adopting them. These will prove to be useful for research ethics review committees and constitute an important aspect of the ethical reflection activities, described above. As noted in Section 5. Regulatory frameworks, the appropriate governmental and/or institutional bodies will establish the requirements for regulatory compliance. A robust ethical inquiry informed by a current understanding of the literature on trial design, relevant precedents, and current government policy at field sites will enhance a GMM research group's ability to develop appropriate protocols and anticipate the concerns of regulatory authorities.

Phase 4

When GMM strategies mature into widespread public health initiatives, it is likely that the responsibilities for implementing these technologies will shift to the relevant local, regional or national public health authorities. Controversy over the fluoridation of public water supplies, regulation of tobacco use and vaccination testifies to the fact that it is not unusual for public health interventions to be undertaken without the explicit approval of all affected parties (Cassidy, 2007; Powles, 2009). They nevertheless have legitimacy when conducted within proper democratic processes and institutions, and with proper mandates. Any public health initiative takes place within the context of legal, regulatory and political institutions that are intended to resolve differences of opinion and to negotiate matters concerning who bears what risks. When public health authorities and the relevant ancillary institutions are functioning well, the responsibility to engage with affected individuals will most likely be transferred to them once it has been established that the technology is

safe and effective. In cases where local or regional institutions are not functioning well, researchers and sponsors may have additional responsibilities related to capacity building and planning with host country agencies, and for maintaining the relationships of trust that were established during the earlier phases of the trials.

4.7 Who should undertake ethics and engagement activities?

The activities described in each section of this guidance framework are material to the successful accomplishment of research objectives. As such they should involve lead researchers and will also often require attention from other members of the project team who are focused on specific tasks. However, there are aspects of each element that may require special skills and training that biologists, medical personnel or public health specialists would not normally be expected to have. As noted above, engagement with affected parties may require specialized knowledge of local culture and institutions. In addition, engagement with third parties is increasingly characterized as requiring skills for creating, maintaining and managing the forums in which discussions, consensus seeking and negotiations can take place (Bäckstrand, 2003; Dietz, Ostrom & Stern, 2003). The abilities and methods for accomplishing these tasks are themselves the focus of ongoing research in communications and governance activities (Brown, Harris & Russell, 2010). Project directors and managers should consult with or contract specialists who can accomplish specialized elements of the ethics and engagement plan (Kreuter et al., 2004; Brown, Harris & Russell, 2010), and allowance made in the project budget for these types of activities. However, researchers should not presume that they can simply turn the ethics and engagement component of the project over to a contractor, as the involvement of project leaders in ethical reflection and engagement, and communication regarding research goals and conduct is vital.

4.8 Capacity-building goals

It is likely that project managers will discover a need for additional training of entomology researchers about ethics obligations in vector biology research. Likewise, there may be a need to train bioethicists and social scientists involved in the project about the unique situations encountered in vector biology research. As discussed above, this is a complex subject where the internationally accepted standards developed for clinical research are not always directly or clearly transferable. Additionally, there may be a need to train institutional and national ethics review committees on the importance and process of ethical review of GMM trials. In both developing and developed countries, ethics review committees often lack vector biologists and awareness of ethical issues in entomological research protocols/proposals. Attempts should, therefore, be made to create awareness of such issues among committee members responsible for approving and providing oversight for the planned trials, and to encourage the committees to seek appropriate expertise when considering GMM research/trials.

Box 4.3 Typical ethical and engagement considerations to prepare for different phases of testing

Phase 1: In the laboratory

- Within the project team and with project advisers, establish ongoing mechanisms for considering the social purpose and public health value of the research and for responding to changing circumstances.
- Develop an initial communications plan with key messages that explain the project and contingency plans for dealing with controversy.
- Initiate public reporting practices, as through publications, project website, etc., to continue throughout the project.
- Conduct preliminary stakeholder mapping; develop plans for discriminating among those who will be affected by the research activities through specific interventions or interactions, other members of host communities who have a stake in the trial, and those who may have legitimate but more distant interests at stake; identify third parties most likely to influence the success of the project.
- Prepare plans for field-site selection; commence discussions with local scientists and community leaders to collect data for decision-making.

Phase 2: Prior to initial field trials

- Develop informational materials appropriate for engagement with government officials, partner institutions, local community and other stakeholders; develop plans for media engagement.
- Conduct more focused assessment of relevant local stakeholders; initiate interactions to build understanding of the project among critical decision-makers.
- Finalize site selection; build knowledge about the host community; develop plans for community authorization and initiate activities to explain the project and elicit community feedback; enact ongoing mechanisms to understand and respond to concerns.
- Secure community authorization and other necessary institutional and government approvals.

Phase 3: Prior to large-scale release

- Review relevant precedents for trial design and broad-scale community engagement.
- Develop locally appropriate communications plans for multiple field sites; consider that large-scale trials will most likely attract global attention and plan to respond accordingly.
- Develop a plan for large-scale engagement, which may require additional mechanisms to interact with and obtain feedback from broad and diverse populations; consider appropriate representational methods to obtain and maintain authorization and ways to evaluate the validity of these methods.
- Take important ethical considerations into account in the development of the trial protocol and ensure adequate oversight of human subjects research by the institutional ethics committee(s); obtain all necessary institutional and government approvals.

Phase 4: Prior to deployment

- Assist agencies in host countries to develop methods for incorporating the technology into their disease control programmes.

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5. Regulatory frameworks

Summary: The release of GMM into the environment will be controlled through the laws and regulations of a nation, state, province, county, or lesser levels of jurisdiction. A number of GMM regulation types, options and levels exist and may have to be addressed during GMM development, including: institutional biosafety and ethics committees; laws and regulations governing human and animal pests, diseases and drugs; laws and regulations pertaining to mosquitoes and threatened, endangered, and protected species in respect to biodiversity; and new laws and regulations, which may be under development, specifically for living or genetically modified organisms (LMOs or GMOs). An important resource for specific country regulations and contacts relevant to GMM is the Cartagena Protocol on Biosafety, Biosafety Clearing-House.

Regulatory agencies will be involved at most phases in the research and development process for GMM and may also be involved in post-implementation surveillance. The mechanisms of regulation may include institutional biosafety and ethics committee approvals, risk assessments, public comment periods, and permits for importation and experiments, and may involve official review by more than one regulatory agency.

GMM regulation is useful both for the scientists involved in their development and for the general public, because it provides a recognized and respected mechanism for protecting human health and rights, livestock, economics and the environment. A thorough, science-based GMM regulatory process that is publicly transparent, without conflict of interest, contains minimal confidential business information, and provides allowance for public stakeholder input, will serve to strengthen public confidence in and acceptance of GMM biotechnologies, their developers, and the government agencies that regulate them.

Regulation controls the release of GMMs into the environment within sovereign states as well as their transboundary movement. Pertinent developments are recorded in Table 5.1. Precedents exist from the regulation of other technologies, including other GM insects of agricultural importance that can inform the formulation of regulatory pathways for GMMs. However, fundamental differences between GMM and other GM technologies must be taken into consideration in order to avoid inadvertently creating barriers to the development of a public health tool of potentially valuable utility. Nonetheless, such considerations must not compromise the safe use of GMMs.

5.1 The purpose of regulations

A regulation is an official rule to manage the conduct of those to whom it applies. Regulations are usually developed from legal interpretations of enacted legislation, laws, or acts of a legislative body and are implemented by government ministries or agencies under the authority of legislation, a law or act. Regulation may be through the laws and official codes of a nation, state or province, county, municipality, tribe or other jurisdictional unit, and/or under the authority of laws and regulations enacted through provisions of a treaty ratified by participating states. A regulatory agency (also called regulatory authority, ministry, regulatory body or regulator) is a public authority or government entity responsible for exercising autonomous authority over some area of human activity in a supervisory capacity.

The purpose of a regulatory agency in regard to GMMs is to ensure that the safety of the public and environment are protected against risks or damage. Risk and, sometimes, benefit, assessments (Section 3. Biosafety) are essential components of the regulatory process. A benefit assessment for GMMs is that of performance or efficacy values for vector and vector-borne disease reduction, without which the increased or continued risk of disease would probably be increased in the absence of alternative effective interventions (Section 1. Introduction). Although performance or efficacy, safety and RA, and public transparency, communication, and acceptance are subsumed as part of the regulatory process, they are not covered in this section since they are discussed elsewhere in this guidance.

Government agency regulation of GMMs could involve more than one regulatory authority and require more than one permit or licence for importation of and research on a GMMs. Further examples of potentially relevant regulations illustrate this issue.

5.2 Biosafety

Institutional biosafety committees (IBCs) are charged by certain laws with the planning and implementation of university and other research facility biosafety programmes for the purpose of protecting the health and safety of all personnel working with potentially hazardous agents. IBCs may be national or may exist at local, regional, state, provincial, or territorial levels of government but they may not exist at an institutional level in some countries. Where they do not exist, they should be part of capacity building by international or foreign aid organizations. IBCs may also draft institutional biosafety policies and procedures and review individual research proposals for biosafety concerns. Concerns relevant to GMMs may relate to the safe handling of recombinant DNA or pathogens perceived to pose a health threat. For example, in the USA, an IBC ensures that research conducted at an institution is in compliance with National Institutes of Health (NIH) Guidelines for Research Involving Recombinant DNA Molecules[‡‡‡‡‡‡‡‡] and the select agent regulations under the Public Health Security and Bioterrorism Preparedness and Response Act of 2002, which authorizes the regulation of the possession, use and transfer of select agents and toxins. The US Federal Select Agent Program[§§§§§§§§] is jointly comprised of the Centers for Disease Control and Prevention/Division of Select Agents and Toxins, and the Animal and Plant Health Inspection Services/Agricultural Select Agent Program. The Federal Select Agent Program oversees the possession, use and transfer of biological select agents and toxins, which have the potential to pose a severe threat to public, animal or plant health, or to animal or plant products. It includes disease agents transmitted by mosquitoes, but does not include *Plasmodium* spp. or dengue virus serotypes. This currently requires registration of facilities including government agencies, universities, research institutions, and commercial entities that possess, use, or transfer biological agents and toxins.

[‡‡‡‡‡‡‡‡] NIH guidelines for research involving recombinant DNA molecules: http://oba.od.nih.gov/rdna_ibc/ibc.html, accessed 25 May 2014.

[§§§§§§§§] USA Federal Select Agent Programme: http://www.selectagents.gov/index.html, accessed 25 May 2014.

5.3 Human subjects

In research, regulations on human subjects generally apply when data will be obtained from living individuals through an intervention or interaction, or identifiable private information will be made available. This will be the case for certain aspects of GMM trials (Section 4. Ethics and public engagement). For example, in GMM trials, regulations on human subjects would apply to the taking of blood specimens to measure epidemiological endpoints (an intervention) or personal opinion surveys to understand concerns about the research (an interaction).

Institutional ethics committees (IECs), also known as institutional review boards (IRBs) or ethical review boards, provide oversight for biomedical and behavioural research involving humans with the aim to protect the rights and welfare of research subjects. Human subjects regulations and IECs were developed in response to notorious abuses carried out in the past in the name of research (Box 5.3).

One role of IECs is to attempt to ensure that human participants in a clinical study understand the facts, implications, and consequences of their participation. Informed consent is the mechanism usually used for this purpose. Informed consent is intended to be a process of communication between an individual contemplating taking part in a study or trial and the physician or scientist administering the study, which results in the patient's decision regarding authorization or agreement. The most important aspect of informed consent is voluntary agreement. In order to give informed consent, the individual concerned must have adequate reasoning faculties and be in possession of all relevant facts at the time of consent. Countries will vary in regard to laws and regulations governing standards of informed consent that are required under common law and statutory authorities. The components of informed consent have been delineated in many venues.*********

For aspects of GMM trials not falling under the definition of human subjects research, mechanisms of community engagement and community authorization (Section 4. Ethics and public engagement) are recommended to communicate goals and risks of the project, and to obtain consent to undertake testing.

*********Informed consent form templates: http://www.who.int/rpc/research_ethics/informed_consent/en/, accessed 25 May 2014.

Box 5.3 The Nuremberg Code, 1949

In medical research, the "Nuremberg Code" from Trials of War Criminals before the Nuremberg Military Tribunals set a base standard following 1947 (Nuremberg Code, 1949). There are 10 points concerning informed consent that are described in the Nuremberg Code and are in National Institutes of Health, Directives for Human Experimentation (NIH, 2010). The Declaration of Helsinki was issued by the World Medical Association (WMA) as a set of ethical principles for the medical community regarding human experimentation. The Declaration is not a legally binding instrument in international law, but instead draws its authority from the degree to which it has been codified in, or has influenced, national or regional legislation and regulations.

The Declaration more specifically addressed clinical research under the term "human experimentation" used in the Nuremberg Code. The operating principles of the Declaration are the following: research should be based on a thorough knowledge of the scientific background (Article 11); a careful assessment of risks and benefits (articles 16 and 17); have a reasonable likelihood of benefit to the population studied (Article 19); be conducted by suitably trained investigators (Article 15) using approved protocols; and be subject to independent ethical review and oversight by a properly convened committee (Article 13). The protocol should address the ethical issues and indicate that it is in compliance with the Declaration (Article 14). Studies should be discontinued if the available information indicates that the original considerations are no longer satisfied (Article 17). Information regarding the study should be publicly available (Article 16). Ethical responsibilities extend to publication of the results and consideration of any potential conflict of interest (Article 27). The interests of the subject after the study is completed should be part of the overall ethical assessment, including assuring their access to care (Article 30). Wherever possible, unproven methods should be tested in the context of research where there is reasonable belief of possible benefit (Article 32).

The International Covenant on Civil and Political Rights (ICCPR, 1976) is a multilateral treaty adopted by the UN General Assembly on 16 December 1966, and implemented on 23 March 1976. It commits its parties to respect the civil and political rights of individuals, including the right to live, freedom of religion, freedom of speech, freedom of assembly, electoral rights, and rights to due process and a fair trial. The ICCPR is part of the International Bill of Human Rights, along with the Universal Declaration of Human Rights (UDHR) and the International Covenant on Economic, Social and Cultural Rights (ICESCR), of which Article 7 states the following: "No one shall be subjected to torture or to cruel, inhuman or degrading treatment or punishment. In particular, no one shall be subjected without his free consent to medical or scientific experimentation."

Sources: Nuremberg Military Tribunals (1949); WMA (1964); NIH (2010).

5.4 GMO regulation

Mosquito pests

The intent or purpose of introducing genetic traits in suppressing mosquito populations could possibly be considered and regulated under the definition of a biopesticide when a pesticide is defined as any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest, which is the USA's Federal Insecticide and Rodenticide Act (FIFRA) definition. Other national pesticide legislation may regulate on the same basis of pesticidal intent.

Mosquitoes are livestock pests as well as human pests. As with existing legislation for crop pests and diseases, many countries have developed legislation to prevent and control outbreaks of livestock pests and diseases, as these issues are in the economic interests of most countries. In the USA, living

modified or GM plants are regulated under legislation intended for the protection of crops under the Plant Protection Act (PPA) of 2000. GM *Drosophila* have been subject to importation and interstate movement permits under this act and more movement permits for GM *Drosophila* have been issued than for all other GMOs combined. GMMs have also been moved and tested in quarantine containment facilities in the past under these same kinds of permits as a courtesy to GMM science and scientists to facilitate their research.

The USA might have regulated GMMs under the Animal Health Protection Act (AHPA) of 2002 because mosquitoes are livestock pests, as well as human disease vectors and pests. The US Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS) administers this Act. The USDA had already regulated GM fruit flies and the pink bollworm under the PPA, and completed the first Environmental Impact Statement (EIS) ever done on any living modified organism (LMO), plant or animal, as well as two Environmental Assessments (EAs) on GM insects that are plant pests in accordance with the US National Environmental Policy Act (NEPA). NEPA includes a number of provisions for public stakeholder participation in the federal decision-making process. There are also litigation precedents in the USA going up to the Supreme Court that have established that the implementation of FIFRA is the equivalent to NEPA.

Legislation pertaining to mosquito control exists in many countries including Australia (Queensland), Malaysia, Singapore, the United Republic of Tanzania and the USA.[†††††††††] This is mainly for the purpose of enforcing control programme requirements, such as the elimination of larval habitats by citizens. According to the Florida Statutes, the creation, maintenance, or causing of any condition capable of breeding flies, mosquitoes, or other arthropods capable of transmitting diseases, directly or indirectly to humans, are prohibited by regulation. In Malaysia, there are laws for the prevention and control of vector-borne diseases. These are: (a) Destruction of Disease-Bearing Insects Act 1975 (Act 154); (b) Prevention and Control of Infectious Diseases Act 1988 (Act 342); and (c) Local Government Act 1976 (Act 171). In Singapore, three pieces of legislation, namely, the Infectious Diseases Act (IDA), the Control of Vectors and Pesticides Act (CVPA) and the Environmental Public Health Act (EPHA) provide broad powers to prevent and control dengue (Seow, 2001). Among those countries in Africa with laws pertaining to breeding of mosquitoes, Tanzanian law goes back to 1913, with legislation governing the breeding of *Anopheles* spp, *Aedes*, spp. and, more recently, *Culex quinquefasciatus*.

GM animals

Genetic modifications to animals are generally for the purpose of affecting their physiology or biology in ways to provide economic or health benefits. Animal drugs or pharmaceuticals are also human interventions intended to similarly affect or alter animal physiology or biology. Legislation for regulation of animal drugs is presently being used to regulate GM animals, including GM salmon, developed for food and drugs and, more recently GMMs. In the USA, the legislation is the Federal Food, Drug, and Cosmetic Act (FFDCA), and the implementing agency is the Food and Drug

[†††††††††] The 2009 Florida statutes: http://archive.flsenate.gov/Statutes/Index.cfm?StatuteYear=2009&Tab=statutes&Submenu=1 (accessed 25 May 2014).

Administration (FDA) within the Department of Health and Human Services (HHS). GMMs are being regulated in the USA by the FDA's Center for Veterinary Medicine (FDA-CVM) under FFDCA as animal drugs.

Environmental protection

Many countries have enacted legislation with regulation by environmental and/or fish and wildlife management agencies for the protection of certain species against adverse effects from human activities. Legislation also exists to protect species that have become threatened or endangered due to human action resulting in potential extinction. Where other regulatory agencies do not have authority because the nature of a LMO may not clearly fit their regulatory scope, environmental agencies may have regulatory purview because of potential adverse impacts on protected species and species diversity in the environment. In this same regard, regulation by other agencies may require endangered and threatened species impact analysis as part of their regulatory process, as is presently required in some countries, including the USA. The Convention of Biological Diversity (CBD, 2012)[‡‡‡‡‡‡‡‡‡] and the Cartagena Protocol on Biosafety[24] are examples of treaties or covenants applying to GMOs/LMOs and are based on protection of species diversity.

Some countries, such as Brazil, Kenya, Malaysia, Nigeria and Panama, have developed specific legislation for LMOs that is based on GM plant experience, but includes other LMOs. Such legislation is usually derived from the CPB, described in Appendix 1. New legislation may require a new regulatory agency to be established or may draw on other agencies or nongovernmental sources for scientific, regulatory, and enforcement expertise. In some countries, this approach may result in either biotechnology implementation delays or possibly regulatory decisions compromised by inadequate science assessment capacity and conflict of interest.

Regulation of GMMs with drive systems capable of autonomous transboundary movement or even movement by inadvertent human transport may invoke regulatory processes of adjacent countries. Gene drive systems are designed and intended to spread throughout an ecozone regardless of political boundaries. If it is known or expected that introduced traits will have transboundary effects, then the need for multilateral regulatory approval by all countries, not separated by species barriers, subject to introduction of a specific GMM should be considered. To engage a multilateral regulatory process may involve international agreements, treaties, covenants, conventions, protocols, or county approvals prior to introduction to one country within a contiguous ecozone. International organizations, such as WHO, may be best suited to provide leadership in a multilateral/international regional regulatory process for deploying GMMs intended to spread widely (see Appendix 2 for further discussion).

‡‡‡‡‡‡‡‡‡ Convention of Biological Diversity: http://www.cbd.int/convention/, accessed 13 June 2014.

Table 5.1 Recent regulatory and biosafety development chronology relevant to the testing and implementation of modified vector insects

Year	Development	Relevance	Website
2000	Cartagena Protocol on Biosafety to the International Convention on Biological Diversity	Established Biosafety Clearing-House for information on national biosafety regulations and contacts	http://bch.cbd.int/
2001–2007	African Model Law on Biosafety	African Union drafted a model legal instrument for developing national biosafety legislations in 2001 that was endorsed by the African Ministerial Conference on Science and Technology in November 2007; several African countries have now approved national biosafety laws	http://hrst.au.int/en/biosafety/modellaw
2002–2004	WHO-TDR Technical Consultations on GM Vectors	Began the process of defining requirements for testing and implementation of GM vectors	http://www.sciencemag.org/content/298/5591/119.full
2002–2007	International Project on LMO Environmental RA Methodologies	Identified and developed scientific methodologies and teaching tools that can be used for environmental RA and management of transgenic plants, in accordance with the Cartagena Protocol on Biosafety and other international agreements	http://www.gmoera.umn.edu/
2005	International Plant Protection Convention (IPPC) Guidelines for the Export, Shipment, Import and Release of Biological Control Agents and Other Beneficial Organisms	IPPC approved international standards for RM related to biological control agents capable of self-replication	https://www.ippc.int/sites/default/files/documents//1323944456_ISPM_03_2003_En_2011-12-01_Refor.pdf
2006	Daegu Protocol on New Technologies for Pest and Disease Control	International effort initiated to establish guidance for regulation of new biotechnologies related to crop pests and human disease vectors	http://www.biopesticide.ucr.edu/daegu/daegu.html
2006	USDA Environmental Assessment on field release of genetically engineered pink bollworm	USDA Animal and Plant Health Inspection Service announced a final environmental assessment and finding of no significant impact to issue permit for confined field trial in southwest US of transgenic plant pest modified to contain fluorescent marker gene	http://www.scribd.com/doc/2810616/Notice-Environmental-statements-availability-etc-Pink-bollworm-genetically-engineered-to-express-green-fluorescence-as-marker-field-trial
2007	NAPPO Guidelines for Importation and Confined Field Release of Transgenic Arthropods in NAPPO Member Countries	NAPPO approved regional standard to provide guidance in use of transgenic arthropods while protecting plant health	http://www.nappo.org/en/data/files/download/Standards/RSPM27-22-10-07-REV%20RL-e.pdf

Year	Development	Relevance	Website
2007	WHO-TDR supported regional centres in Africa, Asia, and Latin America for training in biosafety assessment for human health and the environment of the use of GM disease vectors	Capacity building through a series of training courses targeting researchers, policy-makers, regulators, etc., in developing countries for decision-making on regulatory frameworks, biosafety, RA, and ethical, social and cultural (ESC) issues related to use of GM vectors	http://u-bamako.ml.refer.org/IMG/pdf/Call_for_TDR_Biosecurity_Course_Mali_2008.pdf
2008–2013	WHO-TDR MosqGuide project	Development of best practices for the use of GM mosquitoes, to be used as guidance for decision making in disease endemic countries	http://www.mosqguide.org.uk/
2008	FNIH-supported working group on contained field trials of vector mosquitoes engineered to contain a gene drive system	Development of guidance for the conduct of Phase 2 contained field trials for GM mosquitoes with self-limiting or self-sustaining gene drive	http://www.liebertonline.com/doi/pdfplus/10.1089/vbz.2007.0273
2009	USDA Environmental Impact Statement on Use of Genetically Engineered Fruit Fly and Pink Bollworm in APHIS Plant Pest Control Programmes	USDA Animal and Plant Health Inspection Service, in cooperation with several states and foreign countries, conducts a full RA of GE fruit fly species and pink bollworm for use in various applications of the sterile insect technique (RIDL), determines it to be an environmentally preferable alternative to current technology, and announces intention to integrate use of GM insects into agency invasive plant pest control programmes	http://www.aphis.usda.gov/plant_health/ea/downloads/eis-gen-pbw-ff.pdf
2009	WHO-TDR and FNIH-sponsored technical consultation on progress and prospects for the use of GM mosquitoes to inhibit disease transmission	Reviewed current status and requirements for future development of GM mosquitoes for malaria and dengue control; initiated development of a guidance framework for the evaluation of GM mosquitoes including quality standards for assessing safety, efficacy, and ESC considerations (in progress).	http://apps.who.int/tdr/svc/publications/training-guideline-publications/gmm-report
2010	Ad hoc Technical Expert Group on Risk Assessment and Risk Management under the Cartagena Protocol on Biosafety	Developed a roadmap and guidance on RA and RM of GMOs to supplement Annex III of the Cartagena Protocol, with a special section on living modified mosquitoes	http://www.cbd.int/doc/meetings/bs/bsrarm-02/official/bsrarm-02-05-en.doc
2010–2013	EFSA projects relevant to GM insects	Environment Agency Austria, International Atomic Energy Agency (IAEA) and the University of Bern produced a scientific/technical report "Defining environmental risk assessment criteria for GM insects to be placed on the EU market" Guidance on the environmental RA of GM animals	http://www.efsa.europa.eu/en/scdocs/doc/71e.pdf http://www.efsa.europa.eu/en/efsajournal/pub/3200.htm

5.5 Regulation in a stepwise research and development process

Regulatory oversight will usually be required in **Phase 1** (Figure 1.1) for importation and possibly interstate/interregional movement permits. Inspections may be conducted to assess the security of quarantine containment according to established guidelines. Institutional biosafety committees, where they exist, would also be involved at the beginning of this stage. Other regulatory requirements could be for permits to rear mosquitoes and for permission to work with human disease vectors and the disease agents, if applicable, in the regulatory jurisdictions where the research is to be conducted. Provisions for surveillance and monitoring for escaped GMMs also should be part of the regulatory requirements at Phase 1 because of possible containment failures, since mosquitoes are small and mobile. Regulation at this stage of research and development should also provide for emergency control or mitigation measures to eliminate escaped GMMs through proven means, such as pesticide applications. International biotechnology product movement permits and quarantine systems are already established in many countries for movement of living plant and animal agents that may become pests.

Physically, physiologically and ecologically confined field trials in **Phase 2** should require regulation in which a RA (Section 3. Biosafety) or other similar environmental assessment is conducted and documented to supply scientific rationale and evidence that the confinement will provide the expected degree of assurance that the GMMs will not escape into the surrounding environment and become established and spread or result in spread of the genetic construct(s) into native sexually compatible species. Provisions for surveillance and monitoring should also be part of the regulatory requirements at this phase. Regulation should also provide for emergency control or mitigation measures to eliminate escaped and established GMMs and constructs through proven methods. Clear distinctions should be made between physically and ecologically confined field trials to define what each means relative to the inadvertent dispersion of the GMMs because there would most likely be different regulatory requirements according to the degree of containment or confinement provided.

Open release trials under **Phase 3** should require regulatory RA or other similar environmental assessment documentation to provide a scientific rationale and evidence that the genetic construct(s) are either self-limiting or self-mitigating, and if not 100% self-limiting, that the releases will not then introduce genetic constructs into indigenous wild populations of vectors that may result in increased biological fitness, increased or broadened disease vector capacity, or increased human and animal nuisance impacts. The regulatory RA requirements for open release would be commensurately more stringent than for confined or contained trials in which escape is prevented by physical, physiological, or other barriers. For constructs that are intended to spread within a vector population for the purposes of population suppression or reducing capacity to transmit diseases, there likewise should be regulatory requirements to establish scientifically that the genetic construct(s) do not otherwise increase biological fitness, broaden vector capacity to other disease agents, or increase human and animal nuisance impacts. In case of failure to perform as expected or required, appropriate control or mitigation measures need to be available to eliminate escaped and established GMMs. When transboundary movement to adjacent countries or states with separate regulatory jurisdiction is expected or intended, then prior to the release of GMMs with genetic constructs capable of expanding in a vector population, the regulatory requirement of the countries

or states into which animals containing the transgene may move also needs to be addressed (see discussion of transboundary movement under paragraph 5.6.4 below). Phase 2 and/or 3 also will also require assessment of impact on non-target and beneficial species and include species that are threatened or endangered in the environment. Satisfactory completion of Phase 3 trials may result in regulatory approvals for programmatic implementation and no longer require regulatory supervision for post implementation when all safety-testing parameters are satisfied.

In **Phase 4**, post implementation surveillance regulation, when required, should be intended, designed and implemented to detect movement and introgression of the genetic construct within vector populations and detect unintended changes in vector biology that may result in changes in biological fitness, adverse changes in vectorial capacity, and changes in nuisance impacts. In case of failure to perform as expected or required, emergency control or mitigation measures need to be available to eliminate escaped and established GMMs.

5.6 Additional considerations pertinent to GMM regulation

5.6.1 Public consultation

Regulatory decision-making should include opportunities for public consultation. In many cases, this is mandated within the national regulatory process. For example, in the USA, agencies are required to make efforts to provide meaningful public involvement in their processes under NEPA.[§§§§§§§§§] This principle is also applied in certain multinational agreements. The Cartagena Protocol specifies that Parties shall promote and facilitate public awareness, education and participation, and ensure that the public has access to information on LMOs that may be imported, and shall, in accordance with their respective laws and regulations, consult the public in the decision-making process. The United Nations Economic Commission for Europe (UNECE) Convention on Access to Information, Public Participation in Decision-Making and Access to Justice in Environmental Matters[**********] likewise establishes a number of rights of the public (individuals and their associations) with regard to transparency, consultation and access to justice.

Decision-makers must be able to weigh all the evidence that they receive with regard to relevance and quality. An example is provided in the *WHO Handbook for Guideline Development* (WHO, 2012), which takes into account factors such as the comprehensiveness of the materials, the method by which risk of bias was assessed, the method by which the data were presented, and the similarity of results from different studies.

5.6.2 Litigation

Regulation by litigation may occur when the regulation does not have sufficient basis in law, or is flawed by RA that does not meet Good Laboratory Practice and refereed publication standards or by legally required administrative procedures. Litigation or lawsuits, court injunctions, court orders, fines and penalties may then drive the regulatory process, usually after actions have occurred. There have been several such lawsuits over GM/living modified crop plants. This is the least desirable

[§§§§§§§§§] National Environmental Policy Act (NEPA). Basic information: http://www.epa.gov/compliance/basics/nepa.html, accessed 25 May 2014.

[**********] Aarhus Convention: http://www.unece.org/env/pp/introduction.html, accessed 25 May 2014.

regulatory outcome for GMMs and may result in the loss or delay of beneficial public health innovation as well as loss of public confidence.

5.6.3 Capacity and institution building as an essential component of an informed regulatory infrastructure

The building of regulatory capacity to evaluate GMMs will be unequivocally important. It may be anticipated that there will be a need to train members of national regulatory authorities on issues relevant to the review of entomological intervention trials. In many countries, members of the national regulatory authority may have a pharmacy or medical background with experience in regulating drugs, vaccines and devices. There is a strong probability that they will be unfamiliar with trials of vector control tools, although there are exceptions (for example, in the United Republic of Tanzania, review of vector control trials is done by the Tropical Pesticides Research Institute).

Moreover, although many developing countries have enacted national biosafety legislation, others still do not have a regulatory framework to deal with GMOs. Even if legislation is present, there may not be a functional system in place to regulate GMMs. If experience with RA and regulation of GMOs exists, GM plants or crops may provide the only precedent. Because most legislation dealing with GMOs assigns regulatory responsibility to a separate national biosafety authority, and because the focus of those authorities will probably have been on GM crops, the composition of those bodies will consist of members who have little experience with the technologies involved in producing GMMs or how to regulate them. Regulatory paradigms set by experience with multinational GM plant or crop corporations may result in high costs and extended indecision on regulatory approvals. Adoption of a strict interpretation of the precautionary approach or principle (Appendix 2) could also mean that regulatory approvals would not be granted until all possible safety issues are resolved, regardless of societal needs and potential benefits. This strict interpretation may be incorporated in capacity building efforts conducted by groups opposed to GM technology.

Thus, it will be critical to begin working with regulators very early on in a GMM project to identify the appropriate regulatory pathway and to initiate proactive communications that will build understanding about the GMM technology as well as the goals and methodologies of the project. There may be a need for additional training in vector biology procedures and/or biosafety to ensure that decision-makers are empowered to competently assess plans for GMM trials and reach definitive and defensible conclusions. These needs must be anticipated, and means to address them must be identified and budgeted for accordingly.

5.6.4 Regulatory precedents for transboundary movement

Transboundary regulatory issues that apply to GMMs have been raised because mosquitoes are mobile. For example, the anthropophilic *Aedes aegypti* vector of dengue and other diseases has been spread by humans worldwide wherever suitable habitats exist, especially with increasingly favourable peridomestic habitats provided by ever increasing human urbanization. Thus, RA and RM plans should take into account the possibility that GMMs that are not 100% sterile may move autonomously across political borders into suitable habitats that are contiguous, or even into regions separated by geographical or biological barriers due to human travel and transport.

The general consensus of international conventions that address transboundary movement of GMOs or exotic agents, and that therefore may apply to GMMs, is that prior to release into the environment or implementation, there should be a notification and a bilateral or multilateral consultative process with other countries to which the GMMs may spread. With respect to GMMs that are disease vectors, this could be within the context of a collaborative process for control of the vector.

Relevant conventions that address transboundary movement include the following:

- The World Trade Organization (WTO) Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement) (WTO, 1994), Articles 3, 5 and 6;
- The Convention of Biological Diversity, (CBD, 2014),[35] Articles 3, 4, 5, 14 and 17;
- The Cartagena Protocol on Biosafety, Articles 4, 6, 8, 14, and 19;
- The International Plant Protection Convention (IPPC) Article 7 (International Cooperation) and IPPC International Standards for Phytosanitary Measures (ISPM), Nos. 3 and 11;††††††††††
- Code of Conduct for the Import and Release of Exotic Biological Control Agents;‡‡‡‡‡‡‡‡‡‡
- The ASEAN Agreement on the Conservation of Nature and Natural Resources, Article 3 (ASEAN, 1985);
- The Convention of Conservation of Nature in the South Pacific, Article V;
- The Convention for the Conservation of Biodiversity and the Protection of Wilderness Areas in Central America, Article 24;
- The International Health Regulations, as amended, 1982.

Countries who are Parties to such conventions must develop their own regulations to implement the requirements. The Cartagena Protocol describes an Advance Informed Agreement process that would apply prior to the first intentional transboundary movement of GMMs intended for environmental release in the receiving country (Article 7, paragraph 1). An example of how this provision has been implemented within Europe is found under Regulation (EC) No 1946/2003§§§§§§§§§§ of 15 July 2003 of the European Parliament and of the Council on transboundary movement of genetically modified organisms Official Journal L287 of 05.11.2003.********** This regulation "aims to set up a common system for notifying and exchanging information on transboundary movements of GMOs to third countries. The ultimate goal is to ensure that movements of GMOs that may have adverse effects on the sustainable use of biological diversity and on human health take due account of the environment and human health."

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†††††††††† International Standards for Phytosanitary Measures: https://www.ippc.int/core-activities/standards-setting/ispms, accessed 22 June 2014.

‡‡‡‡‡‡‡‡‡‡ Code of conduct for the import and release of exotic biological control agents. FAO Corporate Document Repository, Report of the Conference of FAO, 28th Session: http://www.fao.org/docrep/x5585e/x5585e0i.htm, accessed 25 May 2014.

§§§§§§§§§§ EUR-Lex. Regulation No. 1946/2003: http://eur-lex.europa.eu/smartapi/cgi/sga_doc?smartapi!celexplus!prod!DocNumber&lg=en&type_doc=Regulation&an_doc=2003&nu_doc=1946, accessed 25 May 2014.

********** Summaries of EU legislation. Transboundary movement of genetically modified organisms: http://europa.eu/legislation_summaries/agriculture/food/l28119_en.htm, accessed 25 May 2014.

5.6.5 Precedents from biocontrol and other areas

The most relevant examples of multilateral collaborative transboundary efforts come from the field of biocontrol. One such success was the introduction the parasitic wasp, *Epidinocarsis lopezi* of the cassava mealybug, *Phenacoccus manihoti,* in Africa (Neuenschwander & Herren, 1988). The parasite was released in more than 50 sites and by the end of 1986, it was established with good results in 16 countries. National introductions were facilitated by inputs from international organizations to guarantee the safety and efficacy of the introductions, including the International Institute of Tropical Agriculture (IITA), the International Institute of Biological Control (IIBC) and the African Union's Phytosanitary Commission (IAPSC). The IAPSC did not make blanket decisions for member countries and releases were national decisions, once imported into quarantine. The IIBC main concern was to ensure freedom from disease and hyperparasites, while IITA assisted governments with local production, release and monitoring of parasites. IITA also coordinated a large capacity building element in the programme, which helped create a generation of technical people across Africa with knowledge of both biocontrol and quarantine, and this has been helpful to further biocontrol projects in Africa (Wagge, 2011, personal communication).

Another example of a successful regional programme is the biological control of the hibiscus mealybug, *Maconellicoccus hirsutus* Green, in the Caribbean (Kairo et al., 2000). Examples of regional disease control programmes include the Pan African Tsetse and Trypanosomiasis Eradication Campaign (ADF, 2004) and the Onchocerciasis Control Programme,[††††††††††] both of which contain vector control components.

[††††††††††] Onchocerciasis Control Programme in West Africa: http://www.who.int/apoc/onchocerciasis/ocp/en/, accessed 25 May 2014.

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Alphey L, Beard C, Billingsley P, Coetzee M, Crisanti A, Curtis C et al. (2002). Malaria control with genetically manipulated insect vectors. *Science* 298:119–21.

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Appendix 1. Examples of national legislation and regulation pertaining to GMMs

This appendix provides a brief description of the regulatory framework of several countries that have engaged in or are contemplating GMM research. The most important resource for specific country GMM regulation and contacts is the Cartagena Protocol on Biosafety, Biosafety Clearing-House.[‡‡‡‡‡‡‡‡‡‡‡] Another source of information is the Convention of Biological Diversity, Biosafety Information Resource Centre.[§§§§§§§§§§]

Brazil

In Brazil, Federal Law # 11.105,[**********] of March 2005, is the principal legal framework for biotechnology and provides safety regulation and inspection tools for activities concerning GMOs and their by-products. This law was implemented by the National Biosafety Council (CNBS), provided a new format for the National Biosafety Technical Commission (CTNBio), and established a framework through the National Biosafety Policy (PNB). CNBS is linked directly to the Office of the President of Brazil and is responsible for providing the PNB. The CNBS is responsible for establishing principles and guidelines for the administration of federal agencies that regulate biotechnology. Also, CNBS analyses the socioeconomic impact of the commercial use of GMOs and their by-products and issues the final approval of licences and policies, when deemed necessary.

CTNBio belongs to the Ministry of Science and Technology of the Federal Government of Brazil and is a consulting and deliberating multidisciplinary body that provides technical assistance to support biotechnology decisions at the federal level. CTNBio is responsible for approvals of research and development of GMOs under specific conditions and approval for tests or commercialization of any biotechnology product for human, animal, and plant use. CTNBio must approve every laboratory or facility that intends to manipulate genes for the creation of GMOs prior to operation. The Commission has 27 members that include scientists with biotechnology backgrounds, federal officers, lawyers and other experts.

In order to have a prior analysis before submission to CTNBio, all organizations (university, research institution, and industry) must have an internal Biosafety Commission that does the initial evaluation of the research. After approval at this first level, the research project is submitted to CTNBio. The requirements for approval of commercial products are quite strict and may take years to be accepted, but mainly involve new plant varieties. After approval, the executing organization must periodically report on implementation and provide results to CTNBio.

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‡‡‡‡‡‡‡‡‡‡‡ Convention of Biological Diversity, Cartagena Protocol on Biosafety, Biosafety Clearing-House: http://bch.cbd.int, accessed 25 May 2014.

§§§§§§§§§§ Convention of Biological Diversity, the Biosafety Information Resource Centre (BIRC): http://bch.cbd.int/database/resources/, accessed 25 May 2014.

********** Government of Brazil. Lei No. 11.105, de 24 de Marco de 2005: http://www.planalto.gov.br/ccivil_03/_Ato2004-2006/2005/Lei/L11105.htm, accessed 25 May 2014.

Malaysia

The Biosafety Act (2007)[††††††††††††] (Act 678) established the National Biosafety Board to regulate the release, import, export, and contained use of LMOs, and the release of their products with the objectives of protecting human, plant and animal health, the environment and biological diversity. The Board consists of the following members: Secretary General of the Ministry of Natural Resources and Environment, who is the Chairman, and representatives from the ministries of agriculture and agro-based industry; the Ministry of Health, Ministry of Plantation Industries and Commodities; Ministry of Domestic Trade and Consumer Affairs; Ministry of International Trade and Industry; Ministry of Science, Technology, and Innovation; and not more than four other persons who have the knowledge or experience or both in any of the disciplines or matters relevant to this Act. A Director General is the Secretary of the Board and carries out duties required by it.

The stated functions of the Board are to: decide on all applications; to monitor activities relating to LMOs and products of such organisms; promote research, development, education and training activities relating to biosafety; and establish mechanisms to facilitate the collection, storage and dissemination of data relating to LMOs and products of such organisms and biosafety. The Genetic Modification Advisory Committee has been established to provide scientific, technical and other relevant advice to the Director General.

An application for the approval of any release activity, or any importation of LMOs, or both is submitted to the Director General and is accompanied with a RA, a RM report, and an emergency response plan. The RA and RM reports are in a form prescribed by the Minister and contain an assessment of the risk and adverse effect that such LMOs and products of such organisms will have or are likely to have on human, plant and animal health; the environment and biological diversity; and the proposed measures to be undertaken to prevent, reduce or control the risks and adverse effects that they will have or are likely to have. The emergency response plan provides safety measures and procedures for the protection of human, plant and animal health, the environment, and biological diversity against harm or damage caused directly or indirectly by LMOs or products of such organisms, as well as all necessary measures to be taken in the event of an emergency.

Information on Malaysian biosafety regulations and the National Safety Board decision to approve GMM experimentation can be obtained from http://www.biosafety.nre.gov.my

Mexico

Mexico actively participated in negotiations leading to the Agreement on Biological Diversity and when the Cartagena Protocol on Biosecurity was adopted. The Interministerial Commission on Biosecurity and Genetically Modified Organisms (CIBIOGEM)[‡‡‡‡‡‡‡‡‡‡‡‡] was created by Presidential Decree on the 5 November 1999 (Villalobos, 2006). Under Mexican Federal law, CIBIOGEM functions to: present suggestions to the National Normalization Commission about Mexican official standards

[††††††††††††] Malaysian Biosafety Act 2007: http://science.kukuchew.com/2008/04/09/malaysian-biosafety-act-2007/, accessed 25 May 2014.

[‡‡‡‡‡‡‡‡‡‡‡‡] CIBIOGEM: http://www.conacyt.gob.mx/cibiogem/, accessed 25 May 2014.

for the research, production, trade, import, export, movement, commercial use, and consumption of LMOs; promote, together with the Comisión Nacional para el Uso y Conocimiento de la Biodiversidad (CONABIO) [National Commission on the Use and Knowledge of Biodiversity], the establishment of a data bank on the presence and distribution of native species related to LMOs, and monitor mechanisms and evaluate the environmental impact, and the impact on human and animal health resulting from the production and consumption of LMOs; set up an uniform programme for the inspection of LMO research and production plants; and recommend methods for the dissemination of information regarding the benefits, and possible risks of the use and consumption of LMOs to the public.

Additionally, the 1999 decree established the Executive Secretary, the Technical Committee, and the Consultative Council on Biosecurity. The Executive Secretary responsibilities include, but are not limited to: ensuring that laws regarding biosecurity and the regulations of CIBIOGEM are followed by government institutions; registering LMOs and their products and sub-products; establishing and maintaining an up-to-date registry of LMOs; and, establishing and maintaining an up-to-date data bank regarding on the presence and distribution of native species related to LMOs. The activities of the Technical Committee are coordinated by the Executive Secretary of CIBIOGEM, and include preparing and suggesting to the Executive Secretary issues and regulations that have to be submitted for consideration by CIBIOGEM, and, when suggested by CONABIO, reaching agreements with the responsible institutions regarding the performance of risk analyses for LMOs and their products and sub-products.

USA

The USA is not a signatory agent to the CPB and uses its existing national legislation and agencies to regulate LMOs under the Coordinated Framework for Regulation of Biotechnology, (US Office of Science and Technology, 1986). The 26 June 1986 Coordinated Framework for Regulation of Biotechnology exists as an Executive Office of the President, Office of Science and Technology Policy Federal Register 51 FR 23302, announcement of policy notice for public comment, and is a guidance and not a law in the USA.

In summary, this Federal Register notice announces the policy of the Federal agencies involved with the review of biotechnology research and products. This notice includes separate descriptions of the regulatory policies of FDA, EPA, Occupational Safety and Health Administration (OSHA), and USDA and the research policies of the National Institutes of Health (NIH), National Science Foundation (NSF), EPA, and USDA. The agencies will seek to operate their programmes in an integrated and coordinated fashion, and together should cover the full range of plants, animals, and microorganisms derived by the new genetic engineering techniques. To the extent possible, responsibility for product use will lie with a single agency. Where regulatory oversight or review for a particular product is to be performed by more than one agency, the policy establishes a lead agency and consolidated or coordinated reviews.

While certain USDA and US Environmental Protection Agency requirements are in part new, the underlying regulatory regimens are not new. Members of the agricultural and industrial communities are familiar with the general requirements under these laws, which include the Federal

Plant Pest Act, The Plant Quarantine Act, the Toxic Substances Control Act (TSCA), the FFDCA, and the FIFRA. Because this comprehensive regulatory framework uses a mosaic of existing federal law, some of the statutory nomenclature for certain actions may seem inconsistent. Certain laws, such as USDA's Federal Plant Pest Act, require a "permit" before a microorganism pathogenic to plants may be transported between states or imported. Under other laws such as FIFRA, the agencies "license" or "approve" the use of particular products. TSCA requires a "premanufacturing notification". There are also some variations among the agencies in the use of the phrase "genetic engineering." Agencies have agreed to have scientists from each other's staff participate in reviews. Each regulatory review will require that the safety, or safety and efficacy, of a particular agricultural or industrial product be satisfactorily demonstrated to the regulatory agency prior to commercialization.

NEPA imposes procedural requirements, including an open public comment period consultation phase announced in the USA Federal Register, on all Federal agencies to prepare an analysis prior to making a decision to take any action that may significantly affect the environment. Depending on the characteristics of a proposal, an environmental assessment (EA), or a broader environmental impact statement (EIS) may need to be prepared in connection with the release of genetically manipulated organisms. Threatened and endangered species impact assessment is required under the Endangered Species Act (ESA). Federal regulatory decisions regarding permits for GMO environmental release in the USA are subject to either EA for some trials or EIS for large-scale or programmatic use under NEPA. Examples of EAs and EIS can be found for Use of Genetically Engineered Fruit Fly and Pink Bollworm in APHIS Plant Pest Control Programmes (USDA, 2008). The EIS was both a USA and international precedent because it was the first EIS ever done on any LMO in the USA or elsewhere under comparable environmental laws of other countries. EA and/or EIS environmental documentation required in the federal decision-making process must provide for alternatives so that different approaches may be considered besides the preferred or proposed alternative. The Record of Decision for the final EIS on Use of Genetically Engineered Fruit Flies and Pink Bollworm authorized the development and use of these genetically engineered insects in SIT for USDA/state cooperative plant pest eradication and control programmes. Field release testing of GM pink bollworm has been conducted in the USA. However, further large-scale implementation has not yet occurred, although an irradiated GE pink bollworm strain expressing a fluorescent marker gene was used by the APHIS Plant pest programme (under permit), to mitigate accidental escapes of fertile moths around the SIT pink bollworm mass-rearing facility and to assess moth dispersal.

Although the USA regulatory route appears straightforward for GE insects that are plant pests, the route for non-plant pest species, such as GE mosquitoes, has been less clear. Although USDA-APHIS have experience with the regulation of GE insects, the two of the relevant statutes under which USDA-APHIS operates are the Plant Pest Act and the Animal Health Protection Act. Clearly, GE mosquitoes are not plant pests and therefore could only be regulated by USDA-APHIS under the Animal Health Protection Act, which prohibits the importation or entry of any animal that is deemed to disseminate any pest or disease of livestock within the USA. Mosquitoes are known to transmit diseases to livestock, and USDA-APHIS might be involved in their regulation from that standpoint. However, when Oxitec Ltd, a UK based company, submitted an application to USDA-APHIS for the import and field release of GM *Aedes aegypti* for dengue control in 2010, USDA-APHIS decided that

they had no jurisdiction for the regulation of GM *Aedes aegypti* as there was no animal health risk. The FDA-CVM emerged as the lead agency with authority under the FFDCA. Their authority comes from the definition of a drug under FFDCA for GE mosquitoes as "*articles intended for the use in the diagnostic, cure, mitigation, treatment or prevention of disease in man and other animals*" and *"articles intended to affect the structure or any function of the body of man or other animals"*. The recombinant DNA construct when expressed in a GM animal including mosquitoes, meets the definition of a drug in the FFDCA. Under this statute, FDA–CVM therefore become the lead agency, under the coordinated framework for all GE animals requiring pre-market approval. However, FDA-CVM has indicated in their Guidance document (FDA, 2011), that they intend to exert enforcement discretion for certain categories of GE animal. These include: (1) GM animals of non-food-species that are regulated by other government agencies or entities, such as GM insects being developed for plant pest control or animal health protection, and that are under APHIS oversight; and (2) GM animals of non-food species that are raised and used in contained and controlled conditions such as GM laboratory animals used in research institutions. The FDA can also exercise enforcement discretion based on the risk profile as it did in the case of the zebra fish (Glo-fish) genetically engineered to express a fluorescent gene and glow in the dark.[§§§§§§§§§§§] When FDA reviews an Investigational New Drug Application (INDA) or a New Animal Drug Application (NADA) it is also subject to NEPA requirements, including a review of environmental risks, as described previously.

European Union

In the European Union (EU), a formal RA is the mechanism by which the risks of the release of a LMO are evaluated. The benefits of such a release are not taken into account within a RA in the EU. The release of a GM insect within any EU member state is controlled by a directive of the European Parliament and of the Council, known as the Deliberate Release Directive (EU, 2001), which regulates the release of all LMOs into the environment. For example, in the United Kingdom, the release of a GM insect is controlled by 'Deliberate Release' regulations transposed from the EU Directive. In the case of a non-commercial release, such as a field trial, the decision to approve release would be made at national level by the United Kingdom's Department for Environment, Food, and Rural Affairs (DEFRA) in consultation with the independent scientific experts of its Advisory Committee on Releases to the Environment (ACRE), which is responsible for assessing the risks of the technology. For a commercial release, there is an initial assessment by one 'lead' member state, which must be satisfied with the information provided before the consultation is opened up to the other member states. At the end of the process, the EFSA would be asked to provide its opinion on any unresolved scientific issue. Member states must then reach a qualified majority to approve any release based on scientific evidence. Should the member states fail to reach a decision, the application then passes to the European Commission, which can approve or deny the application based on the scientific opinion of EFSA. The EFSA has developed *Guidance on the Environmental Risk Assessment of Genetically Modified Animals*, including insects.

[§§§§§§§§§§§] Genetic engineering: http://www.fda.gov/animalveterinary/developmentapprovalprocess/geneticengineering/geneticallyengineeredanimals/ucm113672.htm, accessed 25 May 2014.

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Villalobos VM (2006). The interministerial commission on biosecurity and genetically modified organisms in Mexico. In: Status and risk assessment of the use of transgenic arthropods in plant protection. Proceedings of a technical meeting organized by the Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture and the Secretariat of the International Plant Protection Convention, Rome, 3–12 April 2002. Vienna: IAEA (TECDOC-1483; http://www-pub.iaea.org/MTCD/publications/PDF/te_1483_web.pdf, accessed 25 May 2014).

Appendix 2. Guidance to additional information relevant to GMM regulation

International organizations, treaties and covenants

The World Trade Organization (WTO) Agreements and Public Health; A Joint Study by WHO and the WTO Secretariat (WHO/WTO, 2002). This study explains how WTO Agreements relate to different aspects of health policies. It covers several areas including infectious disease control, environment, and biotechnology. The study explains that countries have the right to take measures to restrict imports or exports of products when necessary to protect the health of humans, animals, or plants. If necessary, governments may put aside WTO commitments in order to protect human life. The study discusses application of biotechnology to foods and potential health effects such as gene transfer from plants to microbial or mammalian cells, transfer of antibiotic resistance, and allergenic effects.

The WTO Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement)********** articles include, but are not limited to the following, which also pertain to autonomous transboundary movement of GMMs:

> Article 1, General provisions – This Agreement applies to all sanitary and phytosanitary measures, which may, directly or indirectly, affect international trade. A sanitary or phytosanitary measure is any measure applied to protect animal or plant life or health within the territory of a member from risks arising from the entry, establishment or spread of pests, diseases, disease-carrying organisms, or disease-causing organisms.
>
> Article 2, Basic rights and obligations – Members have the right to take sanitary and phytosanitary measures necessary for the protection of human, animal or plant life and health.
>
> Article 3, Harmonization – To harmonize sanitary and phytosanitary measures on as wide a basis as possible, members shall base their sanitary or phytosanitary measures on international standards, guidelines, or recommendations. Members shall play a full part, within the limits of their resources, in the relevant international organizations and their subsidiary bodies, in particular the Codex Alimentarius Commission, the International Office of Epizootics, and the international and regional organizations operating within the framework of the International Plant Protection Convention, to promote the development and periodic review of standards, guidelines, and recommendations with respect to all aspects of sanitary and phytosanitary measures.
>
> Article 5, Assessment of Risk and Determination of the Appropriate Level of Sanitary or Phytosanitary Protection – Members shall ensure that their sanitary or phytosanitary measures are based on an assessment, as appropriate to the circumstances, of the risks to human, animal, or plant life and health, taking into account risk assessment techniques developed by the relevant international organizations. In the assessment of risks, members shall take into account available scientific evidence; relevant processes and production methods; relevant inspection, sampling and

********** Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement): http://www.wto.org/english/tratop_e/sps_e/spsagr_e.htm, accessed 25 May 2014.

testing methods; prevalence of specific diseases or pests; existence of pest- or disease-free areas; relevant ecological and environmental conditions; and quarantine or other treatment.

Article 6, Adaptation to Regional Conditions, Including Pest- or Disease-Free Areas and Areas of Low Pest or Disease Prevalence – Members shall ensure that their sanitary or phytosanitary measures are adapted to the sanitary or phytosanitary characteristics of the area, whether all of a country, part of a country, or all or parts of several countries from which the product originated and to which the product is destined.

Article 12, Administration – A Committee on Sanitary and Phytosanitary Measures is hereby established to provide a regular forum for consultations. It shall carry out the functions necessary to implement the provisions of this Agreement and the furtherance of its objectives, in particular with respect to harmonization.

The SPS Agreement, Module 8.1, Genetically Modified Organisms[††††††††††††] recognizes standards developed by the IPPC and the World Organization for Animal Health and applies them to LMOs in respect to the following:

- protection of human or animal life from risks arising from additives, contaminants, toxins, or disease-causing organisms in food, beverages, and feedstuffs;
- protection of human life from plant- or animal-carried diseases (zoonoses);
- protection of animal or plant life from pests, diseases, or disease-causing organisms and;
- protection of a country from damage caused by the entry, establishment, or spread of pests.

Regulations on GMMs should conform to the provisions of this Agreement, such as scientific RA and least trade-restrictive measures.

The WTO Agreement on **Technical Barriers to Trade** (**TBT**)[‡‡‡‡‡‡‡‡‡‡‡‡] allows governments to take appropriate measures if they have a legitimate objective, such as protecting health or the environment.

The CBD (UN, 1992). Since the adoption of the Convention, the Conference of the Parties have initiated national action plans in over 100 countries and raised biodiversity awareness, which led to the adoption of the **CPB**.[24] Mechanisms for implementing the CBD consist of National Biodiversity Strategies and Action Plans (NBSAPs). The articles of the CBD that may pertain to transboundary movement of GMMs include the following:

Article 3, Principle – States have the sovereign right to exploit their own resources pursuant to their own environmental policies and the responsibility to ensure that activities within their jurisdiction do not cause damage to the environment of other states or of areas beyond the limits of national jurisdiction.

[††††††††††††] SPS Agreement Training Module: Chapter 8. Current issues: 8.1 Genetically modified organisms (LMOs): http://www.wto.org/english/tratop_e/sps_e/sps_agreement_cbt_e/c8s1p1_e.htm#LMO, accessed 25 May 2014.

[‡‡‡‡‡‡‡‡‡‡‡‡] SPS Agreement Training Module: Chapter 9. Health and other WTO Agreements: 9.3 Technical barriers to trade: http://www.wto.org/english/tratop_e/sps_e/sps_agreement_cbt_e/c9s3p1_e.htm, accessed 25 May 2014.

Article 4, Jurisdictional Scope – The Convention applies to each contracting party, regardless of whether the effects of their activities occur within or beyond the area of their national jurisdiction.

Article 5, Cooperation – Each party shall, as far as possible and as appropriate, cooperate with other contracting parties, directly or through competent international organizations in respect of areas beyond national jurisdiction.

Article 8, In-situ Conservation – Each party shall establish or maintain means to regulate, manage, or control the risks associated with the use and release of living modified organisms, which are likely to have adverse environmental impacts, taking into account the risks to human health.

Article 14, Impact Assessment and Minimizing Adverse Impacts - Each Party shall introduce appropriate procedures requiring environmental impact assessment of its proposed projects that are likely to have significant adverse effects and allow for public participation. Each party shall promote, on the basis of reciprocity, notification, exchange of information, and consultation; bilateral, regional, or multilateral arrangements within the area under jurisdiction of other states. Each Party shall notify immediately affected states of danger or damage.

Article 17, Exchange of Information – The contracting parties shall facilitate the exchange of information from all publicly available sources relevant to the conservation and sustainable use of biological diversity, taking into account the special needs of developing countries.

The **CPB** is the most significant internationally ratified treaty to influence regulation of GMMs in developing countries. It is a supplementary agreement to the CBD and is an international treaty governing the movements of LMOs. It entered into force in September 2003 when the number of signatory countries reached 50 and it now includes at least 160 nations, including most developing countries. The CPB affirms the precautionary approach contained in Principle 15 of the **Rio Declaration on Environment and Development** and **Annex II** of the **Deliberate Release Directive of the European Economic Community** requiring regulators to consider all potential risks, even when there is scientific uncertainty about their extent or existence. Principle 15 of the Declaration states the following: "In order to protect the environment, the precautionary approach shall be widely applied by States according to their capabilities. Where there are threats of serious or irreversible damage, lack of full scientific certainty shall not be used as a reason for postponing cost-effective measures to prevent environmental degradation".[§§§§§§§§§§§§§]

The precautionary principle or approach is analysed in the published European Commission of the European Communities Communication on the Precautionary Principle (EC, 2000). EU codifications of the precautionary principle are further described in the *Summaries of EU legislation*.[**********]

In the precautionary principle or approach, if an action or policy has a suspected risk of causing harm to the public or to the environment, in the absence of scientific consensus that the action or policy is harmful, the burden of proof that it is not harmful falls on those taking the action. This principle allows policy-makers to make discretionary decisions in situations where there is the possibility of harm from taking a particular course or making a certain decision when extensive scientific

[§§§§§§§§§§§§§] Rio Declaration on Environment and Development, Annex 1, Principle 15: http://www.un.org/documents/ga/conf151/aconf15126-1annex1.htm, accessed 25 May 2014.

[**********] Summaries of EU legislation: http://europa.eu/legislation_summaries/consumers/consumer_safety/l32042_en.htm, accessed 25 May 2014.

knowledge on the matter is lacking. The principle implies that there is a social responsibility to protect the public from exposure to harm when scientific investigation has found a plausible risk, but interpretation has been extended by some to mean that regulatory approvals should not be granted until all possible or theoretical risk and safety issues are scientifically resolved, regardless of societal needs and potential benefits.

A significant provision of Protocol Article 21 is the establishment of the Biosafety Clearing-House (BCH)[42] for the compilation and international exchange of important information on movement and release of GM organisms. This useful database contains information relevant to LMOs and national legislation with some governments having provided their biosafety regulatory frameworks and other pertinent regulatory information including important contacts. The BCH purpose is to (a) facilitate the exchange of scientific, technical, environmental and legal information on, and experience with LMOs; and (b) assist parties to implement the CPB.

The Biosafety Information Resource Centre (BIRC)[43] is an electronic catalogues of biosafety-related publications and information resources including: news services, e-mail list servers, online databases and search engines, reports and case studies, journals, newsletters, and teaching materials (manuals, toolkits, and presentations). Its objective is to increase the accessibility and utilization of available biosafety information and resources for policy-makers, educators, researchers, and the general public.

Whereas national regulations take precedence, aspects of the CPB to be considered for planning of field trials of GMMs are outlined below.

> Protocol Article 4 – The Protocol applies to the transboundary movement, transit, handling, and use of LMOs, taking also into account risks to human health. Under the protocol, a country that wants to export LMOs for intentional introduction into the environment must seek advance informed agreement from the importing recipient country.
>
> Article 6 – The provisions of this Protocol with respect to the advance informed agreement procedure shall not apply to LMOs in transit and transboundary movement of LMOs destined for contained use. Contained use means any operation, undertaken within a facility, installation, or other physical structure, which involves LMOs that are controlled by specific measures that effectively limit their contact with, and their impact on, the external environment.
>
> Article 8 – Pertains to notification and that "The notification shall contain, at a minimum, the information specified in Annex I."
>
> Article 10 – Concerns decision procedures and that decisions taken by the party of import shall be in accordance with Article 15, which addresses risk assessment.
>
> Article 14 – Concerns bilateral, regional and multilateral agreements and arrangements. "The Parties shall inform each other, through the Biosafety Clearing-House, of any such bilateral, regional and multilateral agreements and arrangements that they have entered into."
>
> Article 17 – Concerns unintentional transboundary movements of living modified organisms and emergency measures.
>
> Article 19 – Regarding competent national authorities, states "Each Party shall designate one or more competent national authorities, which shall be responsible for performing the administrative

functions required by this Protocol and which shall be authorized to act on its behalf with respect to those functions."

Articles 8, 10 and 13 and Annex III – Concerns environmental risk assessment, taking into account human health.

Part II of the Final Report of the Ad Hoc Technical Expert Group on Risk Assessment and Risk Management under the CPB[††††††††††††††] on Specific Types of LMOs and Traits, C. Risk Assessment of Living Modified Mosquitoes addresses the following:

- scope: This document focuses on the specific aspects of RA of LM mosquitoes developed for use in the control of human and zoonotic diseases;
- issues to be considered in the RA: effects on biological diversity (species, habitats, and ecosystems); new or more vigorous pests, especially those that have adverse effects on human health; harm to or loss of other species; and disruption of ecological communities and ecosystem processes;
- gene flow: gene flow through cross-fertilisation; horizontal gene flow; and persistence of the transgene in the environment;
- evolutionary responses (especially in target mosquito vectors or pathogens of humans and animals); and
- risk management strategies.

The Nagoya–Kuala Lumpur Supplementary Protocol on Liability and Redress to the Cartagena Protocol on Biosafety concerns the question of what would happen if the transboundary movement of LMOs had caused damage. The negotiators were, however, unable to reach any consensus regarding the details of a liability regime under the Protocol. As a result, an enabling clause to that effect was included in the final text of the Protocol (Article 27), which states:

> The Conference of the Parties serving as the meeting of the Parties to this Protocol shall, at its first meeting, adopt a process with respect to the appropriate elaboration of international rules and procedures in the field of liability and redress for damage resulting from transboundary movements of living modified organisms, analyzing and taking due account of the ongoing processes in international law on these matters, and shall endeavor to complete this process within four years.

In February 1999, the African Group in the CBD and the Organization for African Unity (OAU, now the African Union) began to develop the African Model Law on Safety in Biotechnology. Its first purpose was to provide for a harmonized approach towards biosafety in Africa serving as a model legal instrument for developing national biosafety legislations.[‡‡‡‡‡‡‡‡‡‡‡‡‡‡]

The **IPPC** living modified organisms and pest risk analysis (Devorshak, 2006) discussed the following of relevance to transboundary movement of LMOs. The IPPC is a multilateral treaty with the purpose

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[††††††††††††††] Convention of Biological Diversity, Cartagena Protocol on Biosafety, Biosafety Clearing-House Risk Assessment of Living Modified Mosquitoes: http://bch.cbd.int/onlineconferences/guidancedoc_ra_mosquitoes.shtml, accessed 25 May 2014.

[‡‡‡‡‡‡‡‡‡‡‡‡‡‡] African Union Model Law on Biosafety: http://hrst.au.int/en/biosafety/modellaw, accessed 25 May 2014.

of protecting plants and plant health from the introduction and spread of pests of plants, and to promote measures for the control of plant pests. Biological control agents used to control plant pests fall under the scope of the IPPC. The IPPC is identified in the WTO's SPS Agreement as the international standard-setting organization for plant health, and both the IPPC and SPS Agreement also affirm the sovereign right of all member nations to take necessary measures to protect plant life or health from the introduction and spread of pests. Members of the WTO are legally obligated to base their phytosanitary measures on ISPM developed under the auspices of the IPPC. Like the SPS Agreement and the IPPC, the CPB also requires countries to base measures for LMOs on RA. An open-ended expert working group that met in June 2000 included phytosanitary experts and representatives of the CBD, agreed that organisms that do not pose a threat to plant health (e.g. transgenic mosquitoes) do not fall within the scope of the IPPC.

Provisions of the IPPC that may be relevant to GMM research and implementation include the following.

- IPPC Standards for Phytosanitary Measures (2009)[§§§§§§§§§§§§§§] – Contain guidance that may be useful for adopting and incorporating into national regulation of GMMs, especially pertaining to international movement, release, and RA.
- IPPC ISPM No. 2, Framework for Pest Risk Analysis (2009) – This standard provides a framework that describes the pest risk analysis (PRA) process within the scope of the IPPC. It introduces the three stages of pest risk analysis: initiation, pest risk assessment, and pest risk management.
- IPPC Guidelines for the Export, Shipment, Import, and Release of Biological Control Agents and Other Beneficial Organisms (ISPM No. 03) (FAO, 2005) – This standard provides guidelines for RM related to the export, shipment, import, and release of biological control agents and other beneficial organisms. It lists the related responsibilities of contracting parties to the IPPC, National Plant Protection Organizations (NPPOs), or other responsible authorities, importers, and exporters. The standard addresses biological control agents capable of self-replication (including predators, parasites, nematodes, phytophagous organisms, and pathogens, such as fungi, bacteria, and viruses, as well as sterile insects and other beneficial organisms and also includes those packaged or formulated as commercial products. Provisions are also included for import for research in quarantine facilities of non-indigenous biological control agents and other beneficial organisms. The scope of this standard does not include LMOs.

The IPPC includes the following provision in relation to the regulation of biological control agents and other beneficial organisms. Article 7(1) states:

> With the aim of preventing the introduction and/or spread of regulated pests into their territories, contracting parties shall have sovereign authority to regulate, in accordance with applicable international agreements, the entry of plants and plant products and other regulated articles and to this end, may...c) prohibit or restrict the movement of regulated pests into their territories and d) prohibit or restrict the movement of biological control agents and other organisms of phytosanitary concern claimed to be beneficial into their territories.

[§§§§§§§§§§§§§§] International Standards for Phytosanitary Measures: https://www.ippc.int/index.php?id=13399&L=0, accessed 25 May 2014.

Contracting Parties (member nations) should designate an authority with appropriate competencies to be responsible for export certification and to regulate the import or release of biological control agents and other beneficial organisms. The responsible authority should:

- carry out pest-risk analysis prior to import or release of biological control agents and other beneficial organisms;
- ensure, when certifying exports, that the regulations of importing countries are complied with;
- provide and assess documentation as appropriate, relevant to the export, shipment, import or release of biological control agents and other beneficial organisms;
- ensure that biological control agents and other beneficial organisms are taken either directly to designated quarantine facilities or, if appropriate, passed to mass-rearing facilities or directly released into the environment;
- ensure that importers and, where appropriate, exporters meet their responsibilities; and
- consider possible impacts on the environment, such as impacts on non-target invertebrates.

IPPC[**********] ISPM No. 11 addresses risk analysis for quarantine pests including analysis of environmental risks and LMOs. The standard provides details for the conduct of pest-risk analysis to determine if pests are quarantine pests. It describes the integrated processes to be used for RA as well as the selection of RM options. Section S2 of ISPM-11 includes guidance on evaluating potential phytosanitary risks to plants and plant products posed by LMOs. This guidance does not alter the scope of ISPM No. 11 but is intended to clarify issues related to the pest-risk analysis for LMOs.

The **Food and Agriculture Organization (FAO),** Code of Conduct for the Import and Release of Exotic Biological Control Agents.[††††††††††††††] The objectives of this Code are to facilitate the safe import, export and release of exotic biological control agents by introducing internationally acceptable procedures for all public and private entities involved particularly where national legislation to regulate their use does not exist or is inadequate. The Code describes the shared responsibility of the many segments of society involved and the need for cooperation between importing and exporting countries. Standards are described that encourage responsible and generally accepted trade practices, and assist countries to design regulations to control the suitability and quality of imported exotic biological control agents. They also address the safe handling, assessment, and use of such products. Responsibilities are outlined for the entities which are addressed by this Code, including governments, individually or in regional groupings; international organizations; research institutes; industry, including producers, trade associations, and distributors; users; and public-sector organizations such as environmental groups, consumer groups, and trade unions.

All references in this Code to a government or governments shall be deemed to apply equally to regional groupings of governments for matters falling within their areas of competence. Governments should designate the competent authority empowered to regulate or otherwise control and, where appropriate, issue permits for the importation and release of biological control agents. The organization should prepare a dossier for submission to the national authority if the

[**********] International Plant Protection Convention: https://www.ippc.int/, accessed 25 May 2014.

[††††††††††††††] Code of conduct for the import and release of exotic biological control agents. FAO Corporate Document Repository, Report of the Conference of FAO, 28th Session: http://www.fao.org/docrep/x5585e/x5585e0i.htm, accessed 25 May 2014.

organism has already been imported and is currently being held in containment, or if the organism is being imported directly for release. It should include among other information, a RA to estimate the possible environmental impact in the new area in which any possible risks to animal and human health should be identified. This authority should consult with authorities in neighbouring countries within the same ecological area and with relevant regional organizations to clarify and resolve any potential conflicts of interest that may arise between countries. Where problems (i.e. unexpected deleterious incidents) are identified, the authority is to consider and, where appropriate, ensure corrective action is taken and inform all relevant interested parties.

The **NAPPO**, RSPM No. 27, Guidelines for Importation and Confined Field Release of Transgenic Arthropods in NAPPO Member Countries (NAPPO, 2007) is a standard designed to provide guidance to NAPPO member countries (Canada, Mexico and the USA) on importation and confined field release of transgenic arthropods that are known plant pests or have the potential to affect plant health. This includes transgenic arthropods used for biological control and transgenic beneficial arthropods with the potential to affect plant health. Transgenic arthropod species that are not plant pests, but that may pose a phytosanitary risk, because of genetic modification may also be considered under this standard. Issues relating to the potential adverse impact of transgenic arthropods on human and animal health or on biological diversity, and the environment beyond direct and indirect impacts on plant health are not relevant to plant pest issues and fall outside the scope of this NAPPO Standard. Guidance for unconfined release of transgenic arthropods into the environment is not provided in this Standard.

The **International Organization for Biological Control (IOBC)** is an international body involved with transgenic organisms. It has set up a global Working Group on LMOs in integrated plant production.[‡‡‡‡‡‡‡‡‡‡‡‡‡‡‡]

The **World Organization for Animal Health**[§§§§§§§§§§§§§§§] was founded in 1924 and is the world organization for animal health. Some standards developed by the World Organisation for Animal Health (OIE) deal with diseases that have human health and biosafety significance. The OIE has had a Working Group on Biotechnology since 1996. The OIE is principally concerned with animal or livestock health issues that may be associated with GM animals and vaccines. Examples of subjects from OIE sources involving biotechnology include:

- regulations governing veterinary medicinal products containing GMOs in the European Community
- biotechnology applications in animal health and production
- disease-resistant GM animals
- DNA vaccines for aquaculture
- traceability of biotech-derived animals.

[‡‡‡‡‡‡‡‡‡‡‡‡‡‡‡] The International Organisation for Biological and Integrated Control (IOBC). Working Group on LMO's in integrated plant production: http://www.iobc-wprs.org/expert_groups/wg.html, accessed 25 May 2014.

[§§§§§§§§§§§§§§§] World Organization for Animal Health: http://www.oie.int/eng/en_index.htm, accessed 25 May 2014.

Reports, studies and initiatives

The ***Report on Defining Environment Risk Assessment Criteria for Genetically Modified Insects to be Placed on the EU Market*** (Benedict, et al., 2010), written by the Environment Agency Austria, International Atomic Energy Agency and the University of Bern, describes the ongoing developments in the field of GM-arthropods (transformed species, development purposes, and construction of GM-arthropods), and identifies potential adverse effects, as well as methods to investigate them. Crucial arthropod characteristics and necessary baseline information are discussed and the surrogate and modelling approaches evaluated for utility regarding the environmental RA of GM-arthropod. It was concluded that:

> ...the ERA of GM-arthropods should consider various issues regarding the genetic modification, the respective species, and the receiving environment. Potential risks could be identified concerning gene flow and its consequences, effects on target and non-target organisms, management practices and measures, biogeochemical processes and human health. Since potential risks depend on the method used for modification, the purpose of the GM-arthropod and the species itself, it is recommended to follow a case-by-case approach for the ERA of GM-arthropods.

The University of Minnesota **International Project on LMO Environmental Risk Assessment Methodologies** (IPLMO),[**********] is an initiative driven by public sector scientists, most of whom have strong expertise in environmental science, as well as biotechnology, and socioeconomics. The project has identified and developed scientific methodologies and teaching tools (LMO, Environmental RA Project, 2008)[††††††††††††††††] that can be used for environmental RA and management of transgenic plants in accordance with the Cartagena Protocol on Biosafety and other international agreements. IPLMO has also produced a Problem Formulation and Options Assessment Handbook (PFOA), which is a guide to the PFOA process and how to integrate it into an environmental RA of LMOs (LMO, ERA Project, 2007). The PFOA relies upon being transparent, inclusive of all appropriate stakeholders, and rationally informed by the best available science.

The **MosqGuide** project[‡‡‡‡‡‡‡‡‡‡‡‡‡‡‡‡] is funded by WHO-TDR to provide best practice guidance for the deployment of GMMs to control mosquito-borne disease. The project is developing a series of modules dealing with: 1) overview of technology options, social, and regulatory issues; 2) technology research and production phase decisions; 3) pre-deployment country decisions; 4) data handling and environmental monitoring; 5) field survey on attitudes for alternative control methods; 6) curricula for capacity building; and 7) a prototype issues and response model. Also see, MosqGuide Module 7: *Prototype issues/response model for decision making in deployment of GM mosquitoes* – PROPOSAL. This module is for a bio-economic model designed to compare the costs and benefits of various options for malaria control.

[**********] International Project on LMO Environmental Risk Assessment Methodologies: http://bch.cbd.int/database/record.shtml?documentid=11564, accessed 25 May 2014.

[††††††††††††††††] Scientific Methodologies and Teaching Tools: http://www.gmoera.umn.edu/, accessed 22 June 2014.

[‡‡‡‡‡‡‡‡‡‡‡‡‡‡‡‡] MosqGuide project: http://www.mosqguide.org.uk/, accessed 25 May 2014.

The Daegu Protocol[§§§§§§§§§§§§§§§§] proposes regulatory use of an environmental impact assessment (EIA), which may also be known as a strategic environmental assessment or environmental impact statement, according to the country of use, but the purpose and content are generally similar. The guidance is based on the use of EIA documentation and analysis, commonly used in Australia, Canada, North America, the EU, and other countries as a format to provide for public transparency of the process and to meet country government regulatory agency decision-making requirements. The EIA is a document that is developed openly to the public with all available scientific, societal, and stakeholder input. Therefore, the public is provided the opportunity to be informed and comment on decisions to release new forms of biotechnology into the environment before release occurs.

Publications and conference reports

The objective of a meeting on status and RA of the use of transgenic arthropods in plant protection, proceedings of a technical meeting organized by the Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture and the Secretariat of the International Plant Protection Convention (Devorshak, 2006) were to: (1) review the current state of the art of transgenic insect technology; (2) review the current regulatory framework in different countries; and (3) develop a set of guidelines for RA of transgenic insects. Presentations addressed regulatory issues in Argentina, Mexico, New Zealand, the USA and Zimbabwe. The participants concluded that regulatory approval of any transgenic arthropod release will be on this case-by-case basis. With transgenic technology, intellectual property rights of the strains will need to be addressed and the commercial deployment of a transgenic strain will require a complex set of negotiations related to licensing and royalty payments.

From a **Risk Assessment Workshop on Transgenic Insects** held in Kuala Lumpur, November 2008, sponsored by the United Nations Development Programme (UNDP) (Beech et al., 2009), a published paper on deployment of innovative genetic vector control strategies: *Progress on regulatory and biosafety aspects, capacity building and development of best-practice guidance* reviewed current regulation of GMMs in respect to the CPB and individual country needs.

The report from *Progress and prospects for the use of genetically modified mosquitoes to inhibit disease transmission: technical consultation on current status and planning for future development of genetically modified mosquitoes for malaria and dengue control* (TDR, 2009) includes presentations on GMM mosquito technologies in development; community communication and collaboration strategies; public engagement; ecological RA; the transgenic insect environmental impact statement done in the USA; Malaysia's GMM regulatory experience; ethical, legal and social implications; and guidance on GMM testing and development. Regulatory discussions included the trilateral North American Plant Protection Organization standard, ratified in October 2007, the guidance developed by APHIS for permits; and the IPPC standard on deployment of beneficial organisms.

Guidance for contained field trials of vector mosquitoes engineered to contain a gene drive system: recommendations of a scientific working group (Benedict et al., 2008). Section 8, concerns regulation

[§§§§§§§§§§§§§§§§] Daegu Protocol: http://biopesticide.ucr.edu/daegu/daegu.html, accessed 25 May 2014.

of genetically engineered mosquitoes and the following topics were addressed in this section: 1) regulation at different international and national levels; 2) regulatory costs; 3) regulatory impact; 4) international organizations and covenants with potential relevance to genetically engineered vector mosquitoes, including the Cartagena Protocol on Biosafety; 5) addressing regulatory requirements; 6) a proactive approach to regulatory approval; and 7) the USDA, APHIS Environmental Impact Statement on GM insects.

Ethical, social, and cultural considerations for site selection for research with genetically modified mosquitoes (Lavery, Harrington & Scott, 2008) addresses regulatory issues and administrative discussions and concluded the following:

> The prevailing international framework governing the import of GM organisms is the Cartagena Protocol on Bio-safety...Signatories of the Cartagena Protocol (and countries that voluntarily acceded to the terms of the agreement without being formal signatories) are required to establish mechanisms to deal with the import and regulation of GM organisms...The process of determining the key authorities proved to be extremely important, because it provided a clear point of contact (in at least one candidate country) to address detailed questions related to the proposed research...Because all research activities must conform to local laws, it is important to have a clear understanding of what laws deal with the issues in the host country, especially if specific legislation is not yet in force...It is common, under these conditions, for activities related to the import and research with LMOs to be conducted under the auspices of a battery of existing laws, each of which might address specific elements of the proposed import and research uses...Another regulatory issue with important implications for the ethics of research involving GM insects is the requirement for risk assessment before the research, which varies from country to country...This issue may be particularly contentious with respect to environmental impact assessments of the research, which may be a regulatory requirement...and thus may be a formal requirement for the investigators.

A monograph on *Ethical, legal and social issues of genetically modifying insect vectors for public health* (Macer, 2003; 2005) considered a range of ethical issues including animal rights, informed consent, community consensus and environmental viewpoints and states that each community needs to decide its own priorities for methodology of disease policy guidance for ethical genetic engineering and to negotiate with neighbouring countries.

> The approach to genetically modify insects raises few intrinsic ethical issues; however, important environmental and human health concerns need to be assessed before release of any GM insects...The policy that each community adopts should be the product of open dialogue involving all sectors of society. It can be expected that this process will take years and not all communities will endorse genetic control approaches to insect vectors.

An article entitled *When biotech crosses borders* (Angulo & Gilna, 2008) states that rapid action is needed to address loopholes in the international governance of self-dispersing GMOs purposefully released for the management of wild species and diseases.

A letter to the editor in Nature Biotechnology by Marshall (2010) titled T*he Cartagena Protocol and genetically modified mosquitoes* discussed in Part II, C. the *Risk assessment of living modified mosquitoes*, and posed issues and called for a broader discussion on GMMs to address their unresolved biosafety concerns. The author proposed that:

> Perhaps the most important issue inadequately addressed by the guidance document is the ability of mosquitoes engineered with gene drive systems to propagate transgenes across national borders in the absence of an international agreement...The scenario of containment is particularly relevant to GM mosquitoes because, before an open release, trials are being discussed that would take place in field cages exposed to the ambient environment in a location that the species naturally inhabits.

Ostera and Gostin (2011) advocate for new regulatory pathways for research and development of GM arthropods to control disease, including "an international process for rigorous examination of scientific evidence, ethical values, and dispassionate review before genetically or biologically modified arthropod vectors are released into the natural environment." They argue for a balanced approach in any new regulation and that, "if the scientific evidence demonstrates significant disease reduction with low ecological risks, the precautionary principle should not impede meaningful benefits for human health."

A guide to designing legal and institutional frameworks on alien invasive species (Shine, Williams & Gündling, 2000) addresses alien species including those that may be unintentionally introduced and LMOs as a subset of alien species stating that: "it is possible that the release or escape of transgenic, recombinant or novel DNA might have severe and irreversible effects on environmental safety." Potential health impacts are discussed in respect to invasive microorganisms with west Nile virus provided as a recent example. A number of regional international agreements, not previously mentioned, with applicability to GMMs are listed in this chapter including the following:

- the ASEAN Agreement on the Conservation of Nature and Natural Resources (ASEAN, 1985) requires parties to endeavour to regulate and, where necessary, prohibit introduction of alien species (Article 3[3]);
- the Convention of Conservation of Nature in the South Pacific provides that parties shall carefully consider the consequences of deliberate introduction into ecosystems of species not previously occurring therein (Article V [4]);
- the Convention for the Conservation of Biodiversity and the Protection of Wilderness Areas in Central America (Managua, 1992) that requires the adoption of mechanisms to control all exotic species, which threaten ecosystems, habitats, and wild species (Article 24);
- The International Health Regulations (IHR) (Geneva, 1969, as amended, 1982) were adopted by the WHO's World Health Assembly. They are designed to insure maximum security against the spread of infectious diseases to humans.

An *Overview of existing international/regional mechanisms to ban or restrict trade in potentially invasive alien species* (Council of Europe, 2006) summarizes:

> Globalization provides vastly expanded opportunities for species to be transported to new locations through a wide range of pathways. Those alien species that become established and spread can have serious implications, not just for the environment and communities, but also for national trade and development...Prevention measures should be applied to pathways for introduction and be internationally or regionally coordinated.

A report by the PEW Initiative on Food and Biotechnology (2004) made the following statements concerning GM insects: "Genetically modified insects may offer public health and agricultural benefits, but clear regulatory oversight is lacking...It is not clear which legal authority would apply or

whether the agency involved would have the tools it needed to assess and manage the risks involved." This report concludes that the USA federal government "lacks a coordinated regulatory approach to ensure that all GM insects are reviewed for potential environmental, agricultural, food safety, and public health risks and that the international regulatory regime for approving such releases is not at all clear."

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Public comment: *The draft guidance framework was opened for public comment October 29, 2012 to January 7, 2013 on the WHO website* (http://www.who.int/tdr/news/2012/guidance_framework/en/). These comments were provided on a confidential basis; however, we wish to thank all those organizations and individuals who submitted suggestions for improving the final document.

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* Self-identified a professional interest in GMMs.

‡ Self-identified neither a professional nor a commercial interest in GMMs.

≠ Self-identified a commercial interest in GMMs.

¥ Could not be reached in 2014.

Declaration of Interest terms

Professional interest:

- funding from a non-commercial source (e.g. government agency, philanthropic organization) for research on genetically modified mosquitoes;
- service (paid or unpaid) in an advisory capacity to a non-commercial entity on research or regulatory programmes dealing with genetically modified mosquitoes.

Commercial interest:

- a current proprietary interest in a substance, technology or process (e.g. ownership of a patent), to be considered in or otherwise related to the development or testing of genetically modified mosquitoes;
- a current financial interest, e.g. shares or bonds, in a commercial entity with an interest in the development or testing of genetically modified mosquitoes (except share holdings through general mutual funds or similar arrangements where you have no control over the selection of shares);
- an employment, consultancy, directorship, or other position during the past four years, whether or not paid, in any commercial entity which has an interest in the development or testing of genetically modified mosquitoes, or an ongoing negotiation concerning prospective employment or other association with such commercial entity;
- performance of any paid work or research during the past four years commissioned by a commercial entity with interests in the development or testing of genetically modified mosquitoes;
- payment or other support covering a period within the past four years, or an expectation of support for the future, from a commercial entity with an interest in the development or testing of genetically modified mosquitoes, even if it does not convey any benefit to the expert personally but which benefits his/her position or administrative unit, e.g. a grant or fellowship or other payment for the purpose of financing a post or consultancy;
- personal relationship (e.g. spouse, family member) with someone having a financial or commercial interest in genetically modified mosquitoes.

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TDR, the Special Programme for Research and Training in Tropical Diseases, is a global programme of scientific collaboration that helps facilitate, support and influence efforts to combat diseases of poverty. TDR is hosted at the World Health Organization (WHO), and is sponsored by the United Nations Children's Fund (UNICEF), the United Nations Development Programme (UNDP), the World Bank and WHO.